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# Physiological consequences of high water flow on the coral Montastrea annularis (Ellis and Solander, 1786)

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# PHYSIOLOGICAL CONSEQUENCES OF HIGH WATER FLOW ON THE CORAL *MONTASTREA ANNULARIS* (ELLIS AND SOLANDER, 1786)

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Doctor of Philosophy

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By

Lawrence W. Carpenter

2006

**APPROVAL SHEET**

This dissertation is submitted in partial fulfillment of

the requirements for the degree of

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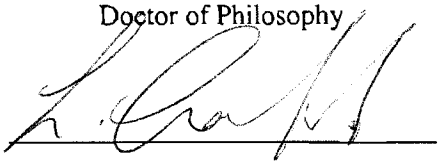

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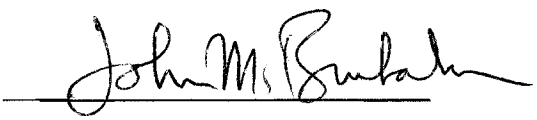

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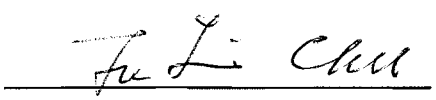

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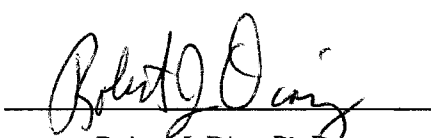

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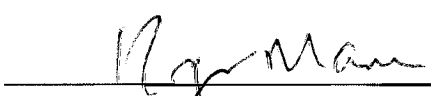
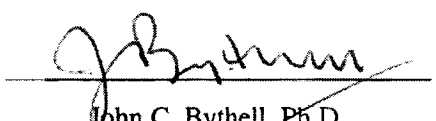


Roger L. Mann, Ph.D.



John C. Bythell, Ph.D.

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## DEDICATION

To my Mother and Father, Serena and Harry Carpenter, to my sister Lesley, my Niece and Nephew Judi and Kneale and course to Sophie and Bella.

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## ABSTRACT

Water-flow is a vital component to the life histories of sessile marine invertebrates and essential to the structure and function of coral reefs. Recent studies have identified water-flow as an asset in the resistance to and recovery from short-term bleaching events of high irradiance and thermal increases. To determine whether the benefits of water-flow scale from the landscape level down to the flow patterns experienced over individual polyps and to quantify potential metabolic consequences, three studies were performed using *in situ* heated bleaching flow-chambers during two saturation missions at the Aquarius underwater laboratory. The first study developed a single coral polyp sampling method and a low-volume protein extraction and quantification protocol. The second study determined and quantified position-effects (upstream and downstream) of enhanced water-flow rates on the organism's photobiology, expressed as Quantum Yield (QY), and within the same experimental set-up, a third study used the regulation and synthesis of the heat shock proteins 70 and 90 (*hsp*70 and 90) as a biomarkers, thus quantifying potential affects of asymmetric flow pattern across the same six coral colonies.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis were used to resolve as little as 87 pg of *hsp*70 per coral polyp. Relatively large amounts of protein ( $\overline{x}$= 77 +/- 9 μg) were recoverable from single coral polyps. *Montastrea annularis* colonies developed and sustained significant spatially asymmetric patterns of stress protein synthesis across the entire coral surface, with upstream sectors expressing more *hsp*70. The mechanism producing this pattern is unclear. We speculate that increased flow may lead to an initial up-regulation in the synthesis of *hsps* by the entire colony followed by a down-regulation in discrete areas of increased hydraulic stress or biochemical energy requirements. The same colonies also developed and sustained significant spatially asymmetric patterns of QY across the entire coral surface, with the upstream side of the colonies exhibiting reduced QY. The mechanism producing this pattern is also unclear. We speculate that increased flow may lead to an up-regulation of photosynthesis by the entire colony through bicarbonate delivery accentuated by the $Q_{10}$ effect. Local down-regulation of the photosynthetic response (decreased quantum yield) might then occur to keep tissue oxygen concentrations within tolerable limits.

This study is the first to investigate asymmetric patterns of flow-modulated stress proteins and photosynthetic regulation, within mounding and flat plate morphologies of *Montastrea annularis*, during and following thermal stress and elevated water-flow. Evidence now exists that following sustained periods of increased flow, irradiance and thermal stress, a yet-to-be determined physiological threshold, if breached, can disrupt metabolic processes *e.g.*, the regulation of molecular chaperone synthesis and/or regulate photosynthetic efficiency. Therefore we propose the existence of a water flow threshold ($FT_{max}$) that operates in concert with *e.g.*, temperature. The threshold is proposed to occur somewhere within the range of 3.0 – 45.0 cm $s^{-1}$. Flow appears not only to accelerate the effects of the co-stressor temperature, but regulates through the direct effects of velocity, exactly where on the coral regulation of stress protein synthesis and photosynthetic efficiency occurs.

Water flow might now be likened to an *agent provocateur* and posited to join the ranks of other bleaching conspirators such as solar radiation and oxygen toxicity, which act synergistically with temperature in lowering the threshold at which thermal anomalies stimulate coral stress.



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# PHYSIOLOGICAL CONSEQUENCES OF HIGH WATER FLOW ON THE CORAL *MONTASTREA ANNULARIS* (ELLIS AND SOLANDER, 1786)

# GENERAL INTRODUCTION

*Coral Reefs*

Coral reef ecosystems account for some of the most developed and ecologically diverse communities on earth and have become centers for socioeconomic development (Hughes and others 2003). These natural assemblages occupy less than 0.2% of the sea floor (Costanza 1997), but provide world-wide economic and ecological services *ca.* $375 billion per annum (Wilkinson 1996; Wilkinson 2000). Over the past three decades reef systems have undergone extensive and unprecedented global degradation and are now considered to be in serious decline from a host of anthropogenic and natural impacts (Jackson and others 2001). Between 20 and 30% of the global coral reef systems are either damaged or in serious decline with a predicted loss of 60% of reef systems by 2030 (Hughes and others 2003; Jackson 2001). Tropical inshore waters are the domains of reef building coral assemblages. Often the geographic limits of these tropical shore lines are defined by the presence of biologically and socio-economically valuable ecosystems (Nybakken 1993; Pandolfi and others 2003). Located in close association with atolls and small islands to continents, the framework of a coral reef is composed of both living and dead corals. An intrinsic element of these tropical shallow water ecosystems are the associated organisms and plants that are major facilitators of resource coupling and recycling that enables coral reefs to maintain high levels of productivity and biodiversity (Hughes 1994; Nybakken 1993). Reef building corals thrive primarily in warm, oligotrophic waters characterized by seasonal variations in physical conditions (Brown and Le Tissier 1995; Leichter and Miller 1999).

*The Coral Organism and Symbionts*

There are two general but distinct groups of coral, *hermatypic and ahermatypic* (Nybakken 1993). Hermatypic corals, also known as hard or stony corals, are the reef builders typically found in tropical regions and may be either solitary or colonial. The distribution of ahermatypic or non-reef building corals can extend from polar to temperate regions. Generally they are incapable of producing extensive developed reef-systems (Barnes 1987; Nybakken 1993). An exception is the deep water coral *Lophelia pertusa* (Linnaeus 1758), with a general depth distribution of 200-1000 m (Fossa and others 2002). These deep coral reef systems can grow to *ca.* 35 m in height, several hundred meters wide, > 10 km in length and some are thought to be *ca.* 8,000 years old. The distribution of coral reefs, are in part limited by the conditions available for zooxanthellae growth and photosynthesis. There are several mutualistic associations within a coral reef community; however, it is the symbiosis between the coral animal and algae, that is a unique feature of hermatypic corals (Barnes 1987; Muscatine 1990). All hermatypic or reef-building corals are obligate associates with endosymbiotic photosynthetic dinoflagellates or zooxanthellae of the genus *Symbiodinium* (Boucher and others 1982; Brown and others 2002b; Rowan and Knowlton 1995). Scleractinians first appeared during the Triassic period (*ca.* 248 to 206 million years ago) when the continent of Pangaea was forcing change to both climate and ocean circulation systems. Scleractinians' rapid ecological success has been attributed to the acquisition of these dinoflagellate endosymbionts (Heckel 1974; Wells 1956). Endosymbiotic algae provide photosynthetic metabolites to their coral host, which enhances calcification rates and coral productivity. In return, the zooxanthellae live in a sheltered microenvironment with

elevated, stable light conditions and nutrient concentrations in the form of inorganic nutrients (high in phosphorous and nitrogen) from the metabolic waste products of the host (Dubinsky and Jokiel 1994; Jokiel and others 1994; Schlichter and Liebezeit 1991). This relationship allows zooxanthellae access to increased concentrations of nutrients not routinely available in oligotrophic waters. The higher productivity of zooxanthellae relative to free-living phytoplankton is a function of the enhanced microenvironment within host cells (Kuhl and others 1995; Schlichter and Liebezeit 1991), with additional benefits for the coral host involving the alga's daytime assimilation of ammonium (Muscatine and Porter 1977). For this mutualistic relationship to have evolved, the benefits to the participating species must first have had to outweigh the costs (as they still must) (Cushman and Beatti 1991).

*Overview of Photosynthesis in Corals*

Photosynthesis is the biological conversion of light energy into chemical bond energy, which is stored in the form of organic compounds. Approximately 40% of the global photosynthesis occurs in the aquatic environment through < 1% of the Earth's total plant biomass (Falkowski and Raven 1997). Carbon is the basis of the biological economy of the Earth, and the majority of this carbon is in an oxidized inorganic form, for example combined with molecular oxygen in the form of carbon dioxide ($CO_2$) or its hydrated or ionic equivalents, that is carbonate ($CO_3^{2-}$) and bicarbonate ($HCO_3^-$). Although these inorganic forms are inter-convertible, they are also thermodynamically stable, contain no biologically usable energy and require a chemical or biochemical reaction to render them available for organic molecular synthesis. To extract energy and use inorganic carbon to build organic molecules, inorganic carbon has to be chemically reduced via the introduction of free energy. There are only a few biological mechanisms extant for the reduction of inorganic carbon, with photosynthesis being the most important and highly studied (Falkowski and Raven 1997).

Photosynthesis can be written as an oxidation-reduction reaction of the general form:

(1) $2H_2\mathbf{A} + CO_2 + \text{Light} \xrightarrow{Pigment} (CH_2O) + H_2O + 2\mathbf{A}$, where **A** can be atom of sulfur (S) or oxygen (O).

The representation of photosynthesis (Eq. 1), has light designated as a reactant. A percentage of the light energy absorbed is ultimately stored in the products of this reaction. All photosynthetic bacteria with the exceptions of prochlorophytes and cyanobacteria, are obligate anaerobes and therefore incapable of producing oxygen. Within these anaerobes compound (**A**) (Eq. 1) can be an atom of sulfur and the pigments are bacteriochlorophylls (Blankenship and others 1995). All other photosynthetic organisms including cynobacteria, eukaryotic algae, prochlorophytes and higher plants are oxygenic, and therefore (Eq. 1) can be modified to:

(2) $2H_2O + CO_2 + \text{Light} \xrightarrow{\text{Chl } a} (CH_2O) + H_2O + O_2$

where Chl *a* is the ubiquitous plant pigment chlorophyll *a*. Eq. 2 implies, that chlorophyll *a* catalyses a series of reactions whereby light energy is harnessed to oxidize water:

(3) $2H_2O + \text{Light} \xrightarrow{\text{Chl } a} 4H^+ + 4e^- + O_2$

yielding gaseous molecular oxygen. The term, *light reaction* in oxygenic photosynthesis is represented by (Eq. 3) and describes an oxidative process, which is a partial reaction where electrons are extracted from water to form molecular oxygen. This process forms the basis for one of two groups of reactions in oxygenic photosynthesis. The second of these processes, the *dark reaction* is the reduction of $CO_2$:

(4) $CO_2 + 4H^+ + 4e^- \longrightarrow CH_2O + H_2O$

Free electrons are not normally available in biological systems; the reactions described by (Eq. 3 & 4) requires the formation of intermediate reducing agents, which have not been shown or discussed. The biological reduction of $CO_2$ is light independent and can therefore proceed both day and night, but cannot proceed spontaneously as it requires a number of enzymes to facilitate the reaction. These so called dark reactions are coupled to light reactions through common intermediates and enzyme regulation. Although variations do exists within the metabolic pathways for carbon reduction, the initial dark reaction whereby $CO_2$ is temporarily fixed to an organic molecule, appears conserved throughout all photosynthetic organism (Falkowski and Raven 1997).

*Measurement and Photosynthetic Capacity of Photosystem II*

Chlorophyll fluorescence is now a widely used indicator to monitor the physiology of symbiotic dinoflagellates within scelaractinian corals and to infer photosynthetic performance. The two main methods currently used to measure changes in chlorophyll fluorescents under dark and light conditions are pulse amplitude modulation (PAM) fluorometry and fast repetition rate fluorometry (FRRF) (Fitt and others 2001; Gorbunov and others 2000; Warner and others 1996).

This study used a Diving Pulse-Amplitude-Modulated (PAM) fluorometer manufactured by Walz Gmbh, Germany. This is a fully submersible fluorometer for measuring the emission of fluorescence, to quantify the efficiency of an organism's photochemistry (Figure 1). The measurement of chlorophyll fluorescence with a pulsed amplitude modulated light was originally developed by (Schrieber and others 1986), is non-destructive, rapid and adaptable to a range of photosynthetic organisms, for example seagrasses, macroalgae and corals. The PAM is simple to use, can be operated *in situ* and programmed to work automatically. For these reasons PAM fluorometry has distinct advantages over other methods for measuring photochemistry, for example $O_2$ evolution.

**Figure 1**. The Diving PAM is a submersible hand held instrument that measures efficiency of photochemistry and photosynthetic rates. The method is non-destructive, rapid and adaptable to a range of photosynthetic organisms. Here an aquanaut (Dr. Jo Gascoigne) makes a measurement of chlorophyll fluorescence from a few polyps of *Montastrea annularis*, the study organism.

This method exploits the fact that while the majority of light captured is used for photochemistry, a lesser proportion is lost as fluorescence and heat. Therefore, a direct relationship is obtained between the efficiency of photochemistry and the re-omitted fluorescence.

***Application of PAM Fluorometry to Corals***

There are three pathways that light energy reaching a photosynthetic organism can follow (Krause and Weis 1991). Light energy may be used in: (1) photosynthesis by the photochemical reactions within the reaction centers of Photosystem II (PSII), for example photochemical quenching, (2) direct competition with photosynthesis as the dissipation of minute quantities of heat, for example non-photochemical quenching, and (3) radiative decay of energy in the form of fluorescence decay, that is the parameter measured by PAM fluorometry (Fitt and others 2001). By measuring the amount of fluorescence emitted, the light energy utilized in the simultaneous photochemical and non-photochemical processes can be quantified (Schrieber and others 1994). If the coral-algal symbiosis are sufficiently acclimated to a dark state, the capacity of the photochemical pathways for light energy absorption are maximized, for example the PSII reaction centers are said to fully opened (Fitt and others 2001). This open state will allow the PS II reaction centers to (almost) entirely absorb the small amount of light energy contained in the first measuring pulse of the Diving PAM, and therefore the resultant initial fluorescence will be minimal. Shortly thereafter a second pulse of intense light saturates the reaction centers, and the resultant fluorescence signal will then be at a

maximal level. The difference between initial fluorescence and florescence maximum, is the fluorescence variable, and is explained further in Chapter 1.

The analysis of chlorophyll fluorescence has allowed further insight into some of the physiological pathways involved during coral bleaching, from increased seawater temperature and the loss a significant number of its symbiotic algae (Warner and others 2002). Likewise, the study of bleached corals during a natural bleaching event revealed a significant decline in PS II function (Warner and others 2002; Warner and others 1999). This decline in photosynthetic efficiency or Quantum Yield (QY) correlated with the loss of a primary PS II reaction-center protein called D1, thereby indicating that dinoflagellates in naturally bleached corals were suffering from chronic photoinhibition. It is the relationship between depressed QY and environmental assaults that this study utilizes to quantify the effects of flow on small colonies of coral.

***Species of interest - Montastrea annularis: (Ellis and Solander, 1786)***

*Montastrea annularis* is the most wide ranging, abundant and intensely studied hermatypic (reef-building) coral in the tropical western Atlantic (Knowlton and others 1992; Leichter and Miller 1999). Found in shallow to intermediate depths *M. annularis* consists of three sibling species that are genetically and morphologically distinct and oft sympatric (Knowlton and others 1992; Lopez and others 1999). The three morphotypes within this complex are, *M. annularis*, formally morphotype I or columnar morph, *M. faveolata*, formerly morphotype II or massive morph, and *M. franksi* (Gregory 1895), formerly morphotype III or bumpy morph (Knowlton and others 1997; Knowlton and

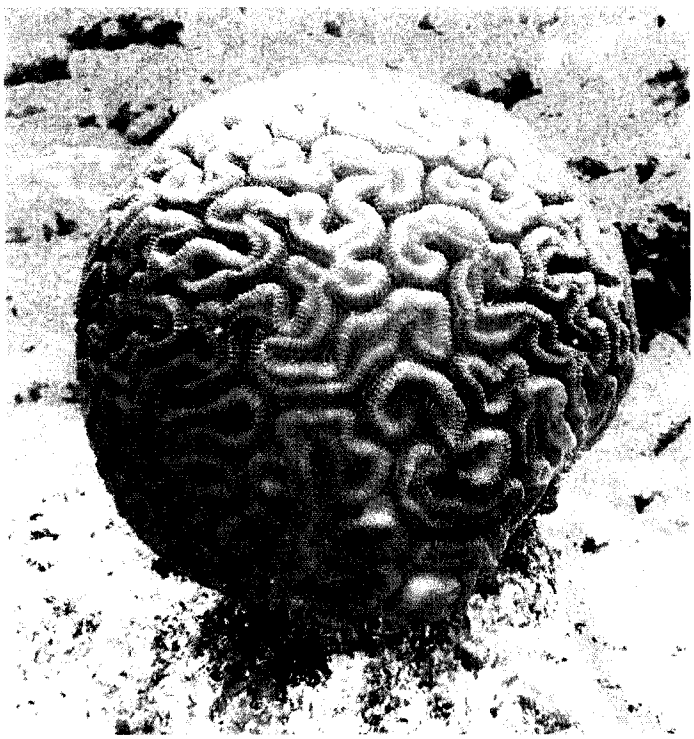
others 1992; Van Veghel and Bak 1993; Weil and Knowlton 1994). Reef-building corals are an obligate mutualistic symbiosis of heterotrophic animals and phototrophic dinoflagellates *Symbiodinium spp.* (Rowan and Knowlton 1995; Rowan and others 1997). Contrary to the widely accepted belief that corals possess only one symbiont (Trench 1993), corals can host a dynamic, multiple-species community of *Symbiodinium* spp. The morphotypes within the *M. annularis* complex appear to act as host for several taxa of *Symbiodinium* spp. (Iglesias-Prieto and Trench 1994; Rowan and others 1997; Toller and others 2001). The three morphotypes differ in their life histories and demonstrate direct evidence of reproductive isolation (Knowlton and others 1997), and a range of inter- and intra-specific distributions of *Symbiodinium* at different reef locations and depths that may also imply past physiological acclimation (Toller and others 2001). The coral samples for this study were collected from a depth range of *ca.* 15 m, insuring the complex morphotypes to be *M. annularis.*

### *Coral Bleaching and Stress*

Coral bleaching was first described in the early 20th century (Boschma 1924; Vaughan 1914; Yonge and Nicholls 1931a; Yonge and Nicholls 1931b). The initial broad definition cited the loss of color as a function of the partial or total elimination of the *Symbiodinium* population and/or the degradation of algal pigments after periods of elevated temperatures (Coles and Brown 2003; Yonge and Nicholls 1931a; Yonge and Nicholls 1931b) Figure 2. These early interpretations suggested bleaching to be the result of a range of stressors impinging on the coral-algal symbiosis and symbiotic associations, for example, light exclusion and starvation. Notwithstanding these early thoughts, coral bleaching is now associated with temperature anomalies which are posited to be the primary stressor causing widespread coral bleaching and associated with the warming of the earth's atmosphere and ocean temperatures (Coles and Brown 2003). For examples of thermally induced stress see (Brown 1997; Brown and others 2002c; Buddemeier and Fautin 1993; Jones and others 1998; Saxby and others 2003) and light saturation (Banaszak and Trench 1995; Brown 1997; Jokiel and Coles 1974). Also, see reviews about coral bleaching (Boesch and others 2000; Brown 1987; Coles and Brown 2003; Fitt and others 2001; Glynn 1991; Wilkinson 2000; Williams and Bunkley-Williams 1990).

**Figure 2**. Example of a brain coral (*Diploria sp.*) having expelled the symbiotic algal cells that give it color, leaving it snow white. **A.** Unbleached. **B.** Bleached (scale bar = 5 cm). Images courtesy of http://coastal.er.usgs.gov.

**A.**



**B.**

Following the loss of the symbiont-algae, the coral tissue can often appear white or bleached (Brown 1997; Glynn 1993; Hoegh-Guldberg 1999). These bleaching events are often accompanied by but not restricted to a reduction in reproductive effort, increased susceptibility to disease, the partial loss of healthy coral at a wide range of scales, and mortality (Brown 1997; Brown and others 2002a). Following extended periods of natural and/or anthropogenic stressors, the distribution of bleached corals can be spatiotemporally patchy at both large and small scales ranging from a few cells at the microhabitat scale, to whole reef tracts following extended periods of low wind, and high thermal and irradiance stress (Brown and others 2002a; Harvell and others 1999; Meesters and Bak 1993; Szmant and Gassman 1990). Considerable variation exists within the thermal tolerance of different species (Coles and Jokiel 1976; Edmondson 1928), and between populations of distant geographic regions (Glynn 1988; Marcus and Thorhaug 1981). These studies in some part explain the variation in the intensity of inter-species bleaching during small local events, and intra-species bleaching patterns between geographically disparate populations during widespread bleaching events. It is difficult, however, it is difficult to cite thermal tolerance as the sole mechanism that explains numerous observations that bleaching can on occasion, be patchy within species at localized scales (Hoeksema 1991; Patterson and Price 1992).

*Adaptive Bleaching Hypothesis*

Three observations with regard to the coral-algal symbioses converged in the early 1990s, coincident with rising concerns over the increasingly widespread and frequent occurrences of coral bleaching. One line of evidence from the molecular study of the algal symbionts in scleractinian corals revealed a high level of diversity in the genus *Symbiodinium* (Rowan and Powers 1991). These findings underpinned the significance of the second line of evidence from previous reports, that different types of symbiotic algae could form metabolically different symbioses with the same host *e.g.*, (Kinzie and Chee 1979). The third line of evidence was derived from reports of variations in bleaching susceptibility, occurrence, and recovery (Jokiel and Coles 1990; Oliver 1985). Striving for a more consistent explanation to combine all three observations, Buddemeier and Fautin (1993) introduced the Adaptive Bleaching Hypothesis (ABH). A basic principle of the ABH is that changing combinations of hosts and symbiotic algae have the potential to create *new* ecospecies *i.e.* a taxonomic species considered in terms of its ecological characteristics and usually including several interbreeding ecotypes that have different environmental tolerances. Diversity and flexibility of partnerships within a host organism, occurring over small spatial scales and short (single generation) timescales, might explain observed differences from one coral population to another *e.g.*, bleaching threshold temperature (Coles and Jokiel 1976; Coles and Jokiel 1977). Buddemeier and Fautin (1993) interpreted these observations as evidence of differences within ecospecies, as what appeared to be a single coral species were actually physiologically different. A competition model was used to explore the parameters underlying the ABH (Ware and others 1996), with the frequency of

*disturbance* being identified as an important factor in determining possible outcomes. Subsequently the ABH was posited in the context of a more conventional, albeit rapid, form of adaptation, based on the potential for somatic mutation to produce genetic chimeras in colonial corals (Abram and others 2003; Buddemeier and others 1997; Rosenberg and Loya 2004).

***Water Flow***

Munk and Riley (1952) determined the importance of water flow in the process of mass transfer to organisms. The range of interactions and associations between marine organisms, specifically lower invertebrates and boundary layers include metabolic processes, feeding, dissolved gas transfer, nutrient exchange and broadcast reproduction, *cf.* numerous examples in (Denny 1988; Vogel 1994). Early studies on coral biology and flow, centered on the hydraulic stress and breakage of corals in high flow environments and resultant modifications of colonial form (Grauss and MacIntyre 1989; Vogel 1994). For examples of sediment removal, growth and prey encounter rates see the following (Patterson and others 1991; Sebens and others 1998; Sebens and others 2003; Sebens and others 1997). With a focus on coral metabolism, studies have now investigated many of the synergistic effects of flow and mass transport in biological and physical interactions (Gardella and Edmunds 2001; Lesser and others 1994; Patterson 1991; Patterson and Sebens 1989). The mass transfer of metabolites and gasses is an essential mechanism for the maintenance, growth and reproduction of many sessile marine organisms (Munk and Riley 1952; Nakamura and others 2003; Thomas and Atkinson 1997). Water flowing across a marine organism will exert forces upon and adjacent to the animal's surface.

These forces generate frictional drag or shear stresses, that is the result of fluid layers *within* a moving fluid gliding along each other or against a static object. This resultant shear stress can mediate the mass transfer of products across the boundary layer (BL), (that is the fluid positioned between the organism and the surrounding body of water), that is oft referred to as *diffusion-limiting* (Patterson and Sebens 1989). The depth or thickness of a particular BL, is a function of water velocity: low-flow generally equates to thick BLs, whilst high-flows equates thin BLs. The combinations of water velocities and an organism's morphological roughness can generate a suite of BL thicknesses. Extending this rationale to low-flow environments would favor organisms with rough morphologies that increase the potential for mass-transfer via a *passive diffusive process*, (that is requiring no energy input from the organism) (Baird and Atkinson 1997; Bruno and Edmunds 1998). Therefore a zero water-flow regime would suggest several problems including diffusion to equilibrium, restriction of metabolic processes and probable death of the organism (Nakamura and van Woesik 2001).

***Heat Shock Proteins***

Within biochemistry, one of the major discoveries in the latter part of the last century was the family of proteins termed molecular chaperones, specifically those known as heat shock proteins (*hsps*). From bacteria to humans, the role of *hsps* appears to be that of critical protein stabilization via the inhibition of nonspecific interactions between and within constituent proteins, that is a pro-active house-keeping mechanism and that of a stress response, that is a re-active mechanism (Ellis 1996; Lindquist 1986). During 'normal' cellular functions, *hsps* fulfill several roles including that of chaperone

activities, translocation and the oligomerization of proteins that is the process of converting a monomer or a mixture of monomers into an oligomer. These aptly named molecular chaperones, include a range of low molecular weight proteins, that is *hsc*70, *hsp60, hsp*70 and *hsp*90, and appear to assist in the folding of proteins, for example when newly synthesized, ensuring the maintenance of tertiary structures. The production and maintenance of these molecular chaperones may also be viewed as a response to stress in the form of a biomarker. A stress response is the function of an interaction between stress and time and therefore the intensity and duration of a stress event, for example thermal increase, will drive the rate and extent a particular stress response will proceed (Hochachka and Somero 2002).

***Induction of Heat Shock Proteins***

Heat shock factors (*hsfs*) are as highly conserved throughout and within phyla as they are integral to the heat shock protein response (Morimoto 1993). Heat shock factors regulate the production of *hsps* and when activated, bind to DNA and induce the transcription of *hsp* RNA (Morimoto and others 1994). Four *hsfs* have been identified in eukaryotic organisms, for example mice, chickens, yeast and humans (Morimoto 1998). The three *hsfs*, 1, 2 and 3, are universally expressed throughout, whilst *hsf*4 appears only in the human heart, skeletal muscle and brain tissue (Morimoto 1998). Each *hsf* appears to respond differently to the stimuli of stress, which suggest each is under a different form of regulation. Heat shock factor 1 appears to be a wide-ranging stress response and binds to DNA in response to elevated levels of heavy metals, oxidative stress and temperature (Morimoto and others 1994). Heat shock factor 2 acts as a developmentally

active *hsf*, and has been studied during mouse spermatogenesis and early embryogenesis, whereas *hsf*3 appears a cell-type specific *hsf*, whereby the response to heat shock is delayed when compared to that of *hsf*1 (Morimoto and others 1994; Robbart and others 2004; Robbart 2002).

The cosmopolitan distribution of *hsps* across widely disparate taxa has led researchers to focus on the role of these proteins in the physiological response of organisms (including scleractinian coral) to thermal stress (Robbart and others 2004). Genes that encode for molecular chaperones appear highly conserved and occur in (nearly) every species in which they have been studied (Feder and Hofmann 1999b; Hochachka and Somero 2002; Hofmann and others 2000). Nonetheless, they appear absent in the stenothermal cnidarian *Hydra oligactis* (Pallas, 1766) an organism adapted to very slight variations in temperature, and in the Antarctic notothenioid fish *Trematomus bernacchii* (Nichols and LaMonte) which has evolved under near-freezing and therefore highly stable water temperatures for some 14 to 25 million years (Buckley and others 2004; Hofmann and others 2000; Somero and DeVries 1967). Typically, an organism will express some level of stress protein synthesis following the exposure to an environmental perturbation. Interestingly, similar breakdown of normal cellular activity is now thought to be the underlying cause of a number of human diseases (Feder and Hofmann 1999a; Hochachka and Somero 2002). Not all stress proteins are induced through thermal stress. Cellular energy depletion, concentrations of chemicals, gasses and toxic substances are also common triggers causing cells to express *hsps* regulation and synthesis. If sufficiently intense, almost all known stressors have been shown to

eventually induce *hsps*, therefore the terms stress protein and stress response are sometimes more appropriate than heat shock proteins and oft used so not to place an emphasis on the single stress of heat (Feder and Hofmann 1999a; Hochachka and Somero 2002). Stress protein regulation is widely used as a biomarker or as an indicator of past and of potential adaptation to future stress-events.

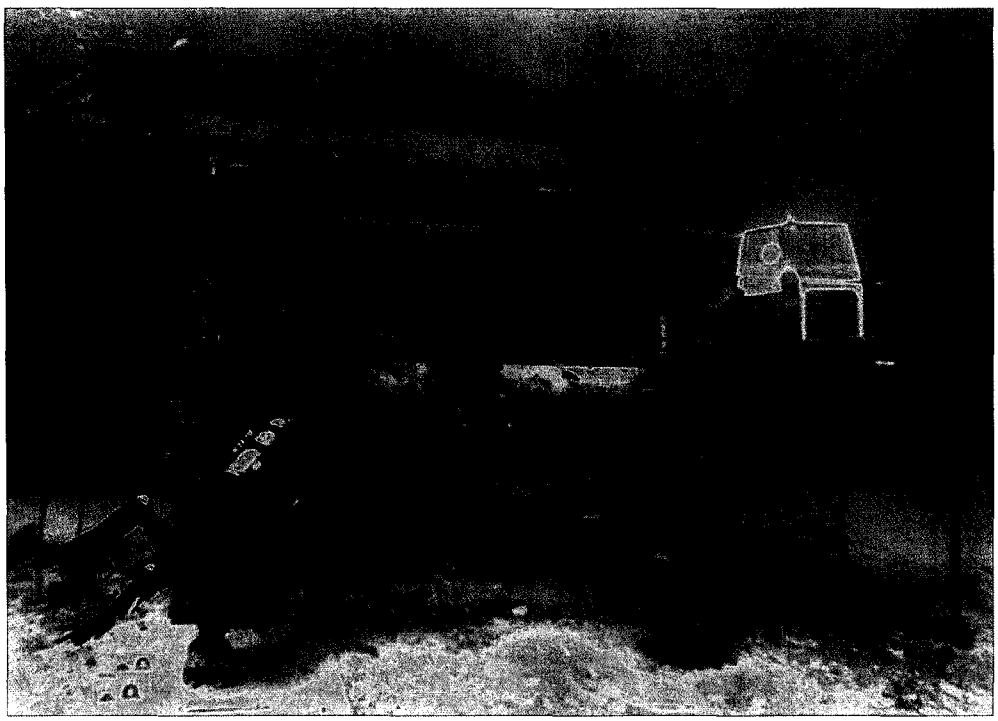
Heat shock protein regulation and synthesis appears well documented in the literature for a number of cnidarians. Six *hsps* were identified in the scyphozoan jellyfish *Aurelia aurita*, when ranges of its developmental stages were exposed to temperature manipulations (Black and Bloom 1984). Two thermo-tolerant species of the hydrozoan, *Hydra*, increased the synthesis of *hsp*60 when exposed to temperature elevation, sodium azide and cadmium chloride; whereas, six thermo-sensitive species failed to produce the same *hsp* (Bosch 1988). For additional (marine) examples in *bivalves* see (Clegg and others 1998; Hamdoun and others 2003), *molluscs* (Tomanek and Somero 2000), *cnidaria* (Bosch 1988; Sharp and others 1997), and *coral* (Black and others 1995; Downs and others 2000; Robbart and others 2004; Sharp and others 1997).

***This Study***

The three studies presented in the following chapters and briefly described below, were performed to spatiotemporally resolve and quantify the affects of an enhanced water flow on photosynthetic efficiency and the regulation of stress proteins. Three novel methods were developed to facilitate the low volume time-series sampling and subsequent extraction and quantification methods required to complete this study. The two *in situ* studies were conducted at the NOAA Aquarius underwater habitat located

within the Florida Keys National Marine Sanctuary at 24° 57.230' N, 80° 27.223' W, during two (ten day) saturation missions in November 2002 and July 2003 (Figure 3). Using *in situ* independently controlled coral microcosms, time-series single coral polyp samples and coincident PAM measurements, We now propose a shift within our understanding of increased flow and the benefits to coral health. The concept of a flow threshold ($FT_{max}$) and its effect on the distribution and level of photosynthetic yield, and the regulation and positioning of *hsp*70, introduces a hitherto overlooked limiting stressor of corals exposed to higher flow conditions. This concept may also occur regardless of other bleaching conditions. Temperature increases form the basis of our current understanding of coral bleaching. It is these thermal excursions that accelerate the impact of any co-stressors, for example salinity and light.

**Figure 3.** Aquarius rests 20 meters underwater, off Key Largo, in the Florida Keys National Marine Sanctuary. Aquarius is an undersea laboratory designed to support research in coastal and ocean resource science and management. The habitat itself, a steel cylinder 3 meters in diameter by 14 meters long, provides 11 cubic meters of living and laboratory space for a six-person crew. The lab is equipped with computers networked to shore, Internet, telephones, radios, and videoconferencing equipment. Image courtesy of NOAA's Undersea Research Center at the University of North Carolina Wilmington.



Within coral bleaching, water flow has ether been ignored or posited as a mechanism for corals to resist and recover from environmental stress (Nakamura and van Woesik 2001; Nakamura and others 2003; West and Salm 2003). The flow-mitigation of oxidative stress has (in part) explained the increased incidence of coral bleaching in areas of low flow, and enhanced recovery in areas of higher water flow (Finelli and others 2006; Gardella and Edmunds 1999). At the level of individual small coral colonies, and assuming a bi-directional rapidly accelerating flow, for example due to bathymetry and wave orbitals, a $FT_{max}$ would cause the rapid cycling of photosynthetic efficiency, therefore relocating the maximum of this metabolic process about the coral. Stress proteins regulation, although highly responsive, does not respond at the same rate as photosynthesis and would, therefore, increase over the entire organism when bathed in such a flow. Both these metabolic processes would impact on a coral's energy budget. Regardless of scale, flow can rapidly relocate metabolic processes within corals, which in turn during periods of high temperature, would accelerate and modulate stress effects.

***Chapter 1. Water-flow Influences the Spatiotemporal Distribution of Photosynthetic Efficiency within Colonies of the Coral* Montastrea annularis *(Ellis and Solander, 1786): Implications for Coral Bleaching***

We measured photosynthetic yield on small patches of polyps within a colony of the scleractinian coral *M. annularis* using *in situ* heated flow chambers. We addressed the central question: Is coral bleaching, as measured by photosynthetic efficiency within a colony, modulated by water-flow? The objective of this chapter was: (i) to establish and maintain six individual coral colonies in self-contained coral microcosms during two

ten-day saturation missions, (ii) to systematically collected time series fluorescence emission data from four spatially distinct coral sectors and (iii) resolve any spatiotemporal affect that an enhanced water-flow may impose on photosynthetic efficiency, across the up and downstream surfaces of individual coral colonies relative to flow. Field observations during bleaching events in Jamaica (1990), Florida (1991) and subsequent flume experiments indicated a preliminary pattern of upstream/downstream asymmetry in bleaching with colonies of *M. annularis* and *M. cavernosa* (Patterson and Price 1992).

***Chapter 2. A Novel Non-Destructive Method of Sampling Live Coral Tissue, Single Polyp-Sample Preparation and Protein Quantification For Assessment of Coral Heat Shock Proteins***

Current coral sampling methods often cleave coral fragments ~ 100 $cm^2$ off live coral colonies that may already be in a stressed or poor condition. Subsequent protein preparation methods then have to further purify the sample to account for contaminants, for example $CaCO_3$ and coral mucus and often process these contaminants in concert with the attached soft tissue. This process invariably leads to contaminated western blots that are difficult to quantify. The main focus of this study was: (i) carry out single polyp time-series sampling on live coral colonies over two field seasons without compromising the colonies or the parallel studies on photosynthetic efficiency, (ii) develop a protein extraction method for low volume (50-100 $\mu$l) single coral polyps and (iii) develop a low volume, high resolution protein quantification method.

## *Chapter 3. Water Flow Influences the Spatiotemporal Distribution of Heat Shock Protein 70 within Colonies of the Coral* **Montastrea annularis** *(Ellis and Solander, 1786): Implications for Coral Bleaching*

Would a high flow environment and a coincident thermal increase result in an asymmetric expression of stress protein synthesis similar to the expression of QY? (*cf.* Chapter 1). The synergistic effects of water-flow and temperature upon stress protein synthesis were investigated. Several stress proteins were studied, the constitutive form, *hsc*70 was investigated to identify any early/initial response, and *hsp70* to identify any up-regulation (increased synthesis) and/or sustained expression of stress protein synthesis similar to that found in other studies. The potential co-expression of *hsp*90 was also studied, given its similar molecular functions. The objective of this study was (i) to establish and maintain six individual coral colonies in self-contained coral microcosms during two ten-day saturation missions, in concert with the requirements for measuring changes in photosynthetic efficiency, (ii) to remove a daily time-series of single coral polyps from different flow environments across the coral's sectors and (iii) following protein extraction and quantification (*cf.* Chapter 2), resolve potential spatiotemporal effects that an enhanced water-flow may impose on the regulation of stress proteins, across the up and downstream sectors of individual coral colonies.

## LITERATURE CITED

Abram NJ, Gagan MK, McCulloch MT, Chappell J, Hantoro WS. 2003. Coral reef death during the 1997 Indian Ocean dipole linked to Indonesian wildfires. Science 301:952-955.

Baird M, Atkinson MJ. 1997. Measurement and prediction of mass transfer to coral reefs. Limnology and Oceanography 42(8):1685-1693.

Banaszak AT, Trench RK. 1995. Effects of ultraviolet (UV) radiation on marine microalgal invertebrate symbioses. I. Response of the algal symbionts in culture and in hospite. Journal of Experimental Marine Biology and Ecology 194:213-232.

Barnes RD. 1987. Invertebrate Zoology, 4th ed. Philadelphia: Saunders College. p 181-200.

Black NA, Voellmy R, Szmant AM. 1995. Heat shock protein induction in *Montastraea faveolata* and *Aiptasia pallida* exposed to elevated temperatures. Biological Bulletin 188:234-240.

Black RE, Bloom L. 1984. Heat shock proteins in Amelia (Cnidaria, Scyphozoa). J. Exp. Zoo/. 230: 303-307. Journal of Experimental Zoology 230:303-307.

Blankenship RE, Madigan MT, Bauer CE, eds. 1995. Anoxygenic photosynthetic bacteria. Dordrecht, Netherlands: Kluwer Scientific.

Boesch DF, Field JC, Scavia D. 2000. ''The potential consequences of climate variability and change on coastal areas and marine resources''. NOAA Coastal Ocean Program( Decision Analysis Series No. 21.).

Bosch TCG. 1988. Thermotolerance and synthesis of heat-shock proteins: These responses are present in *Hydra attenuata* but absent in *Hydra oligactis*. Proceedings of the National Academy of Science 85:7927-7931.

Boschma H. 1924. On the food of the Madreporaria. Proceedings of the National Academy of Sciences 27(13-23).

Boucher DH, James S, Keeler KH. 1982. The ecology of mutualism. Annual Review of Ecology and Systematics 13:315-347.

Brown BE. 1987. Worldwide death of corals - Natural cyclical events or man-made pollution? Marine Pollution Bulletin 18(1):9-13.

Brown BE. 1997. Coral bleaching: Causes and consequences. Coral Reefs 16(Suppl.):S129-S138.

Brown BE, Downs CA, Dunne RP, Gibb SW. 2002a. Exploring the basis of thermotolerance in the reef coral *Goniastrea aspera*. Marine Ecology Progress Series 242:119-129.

Brown BE, Downs CA, Dunne RP, Gibb SW. 2002b. Preliminary evidence for tissue retraction as a factor in photoprotection of corals incapable of xanthophyll cycling. Journal of Experimental Marine Biology and Ecology 277:129-144.

Brown BE, Dunne RP, Goodson MS, Douglas AE. 2002c. Experience shapes the susceptibility of a reef coral to bleaching. Coral Reefs 21:119-126.

Brown BE, Le Tissier MDA. 1995. Mechanisms of bleaching deduced from histological studies of reef corals samples during a natural bleaching event. Marine Biology 122:655-663.

Bruno JF, Edmunds PJ. 1998. Metabolic consequences of phenotypic plasticity in the coral *Madracis mirabilis* (Duchassaing and Michelotti): the effect of morphology and water flow on aggregate respiration. Journal of Experimental Marine Biology and Ecology 229:187-195.

Buckley BA, Place SP, Hofmann GE. 2004. Regulation of heat shock genes in isolated hepatocytes from an Antarctic fish, *Trematomus bernacchii*. The Journal of Experimental Marine Biology 207:3649 3656.

Buddemeier RW, Fauth JE, Ware JR. Acclimation, Adaptation, and Algal symbiosis in Reef-building Scleractinian corals. In: Hartog JC, editor; 1997 16-21 July 1995; Noordwijkerhout, The Netherlands. National Museum of Material History.

Buddemeier RW, Fautin DG. 1993. Coral bleaching as an adaptive mechanism: a testable hypothesis. BioScience 43:320-326.

Clegg JS, Uhlinger KR, Jackson SA, Cherr GN, Rifkin E, Friedman CS. 1998. Induced thermotolerance and the heat shock protein-70 family in the Pacific oyster *Crassostrea gigas*. Molecular Marine Biology and Biotechnology 7(1):21-30.

Coles SL, Brown B. 2003. Coral Bleaching – Capacity for Acclimatization and Adaptation. Advances in Marine Biology 46.

Coles SL, Jokiel PL. 1976. Thermal tolerance in tropical versus subtropical Pacific reef corals. Pacific Science 30:159-166.

Coles SL, Jokiel PL. 1977. Effects of temperature on photosynthesis and respiration in hermatypic corals. Marine Biology 43:209-216.

Costanza R. 1997. The value of the world's ecosystem services and natural capital. Nature 387:253-260.

Cushman JH, Beatti AJE. 1991. Mutualisms: Assessing the benefits to hosts and visitors. Trends in Ecology and Evolution 6:193-195.

Denny MW. 1988. Biology and mechanics of wave-swept environment. Princeton, New Jersey: Princeton University Press. 329 p.

Downs CA, Mueller E, Phillips S, Fauth JE, Woodley CM. 2000. A molecular biomarker system for assessing the health of coral (*Montastraea faveolata*) during heat stress. Marine Biotechnology 2:533-544.

Dubinsky Z, Jokiel PL. 1994. Ratio of energy and nutrient fluxes regulates symbiosis between zooxanthellae and corals. Pacific Science [PAC SCI ] Vol 48(3).

Edmondson CH. 1928. The ecology of an Hawaiian coral reef. Bernice P. Bishop Museum Bulletin 45:1-64.

Ellis RJ. 1996. The "bio" in biochemistry: Protein folding inside and outside the cell. Science 272:1448-1449.

Falkowski PG, Raven JA. 1997. Aquatic Photosynthesis. United States of America: Blackwell Science. 375 p.

Feder ME, Hofmann GE. 1999a. Heat-shock proteins, molecular chaperones and the stress response: Evolutionary and ecological physiology. Annual Review of Physiology 61:243-282.

Feder ME, Hofmann GE. 1999b. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. Annual Review of Physiology 61:243-282.

Finelli CM, Helmuth BST, Pentcheff DN, Wethey DV. 2006. Water flow influences oxygen transport and photosynthetic efficiency in corals. Coral Reefs 25:47-57.

Fitt WK, Brown BE, Warner ME, Dunne RP. 2001. Coral bleaching: Interpretation of thermal tolerance limits and thermal threeholds in tropical corals. Coral Reefs 20:51-65.

Fossa JH, Mortenson PB, Furevik DM. 2002. The deep-water coral *Lophelia pertusa* in Norwegian waters: Distribution and fishery impacts. Hydrobiologia 471:1-12.

Gardella DJ, Edmunds PJ. 1999. The oxygen microenvironment adjacent to the tissue of the scleractinian *Dichocoenia stokesii* and its effects on symbiont metabolism. Marine Biology 135:289-295.

Gardella DJ, Edmunds PJ. 2001. The effect of flow and morphology on boundary layers in the scleractinians *Dichocoenia stokesii* (Milne-Edwards and Haime) and *Stephanocoenia michilini* (Milne-Edwards and Haime). Journal of Experimental Marine Biology and Ecology 256:279-289.

Glynn PW. 1988. El Nino-Southern Oscillation 1982-1983: nearshore population, community, and ecosystem responses. Annual Review of Ecology and Systematics 19:309-345.

Glynn PW. 1991. Coral reef bleaching in the 1980s and possible connections with global warming. TREE 6(6):175-179.

Glynn PW. 1993. Coral reef bleaching: ecological perspectives. Coral Reefs 12:1-17.

Gorbunov MY, Falkowski PG, Kolber Z. 2000. Measurement of photosynthetic parameters in benthic organisms *in situ* using a SCUBA-based fast repetition rate fluorometer. Limnology and Oceanography 45(1):242-245.

Grauss RR, MacIntyre IG. 1989. The zonation patterns of Caribbean coral reefs as controlled by wave and light energy input, bathymetric setting and reef morphology: Computer simulation experiments. Coral Reefs 8:9-18.

Hamdoun AM, Cheney DP, Cherr GN. 2003. Phenotypic plasticity of HSP70 and HSP70 gene expression in the Pacific Oyster (*Crassostrea gigas*): Implications for thermal limits and induction of thermal tolerance. Biological Bulletin 205:160-169.

Harvell CD, Kim K, Burkholder JM, Colwell RR, Epstein PR, Grimes DJ, Hofmann EE, Lipp EK, Osterhaus ADME, Overstreet RM and others. 1999. Emerging marine diseases--climate links and anthropogenic factors. Science 285(5433):1505-1510.

Heckel PH. 1974. Carbonate buildups in the geologic record: A review. In: Laporte LF, editor. Reefs in Time and Space: Selected Examples from the Recent and Ancient: Society of Economic Paleontologists and Mineralogists Special Publication. p 90-154.

Hochachka PW, Somero GN. 2002. Biochemical Adaptation, Mechanisms and Process in Physiological Evolution. New York: Oxford University Press. 466 p.

Hoegh-Guldberg O. 1999. Climate change, coral bleaching and the future of the world's coral reefs. Marine and Freshwater Research 50:839-866.

Hoeksema BW. 1991. Evolution of body size in mushroom corals (Scleractinia:Fungiidae) and its ecomorphological consequences. Netherlands Journal of Zoology 41(2-3):112-129.

Hofmann GE, Buckley BA, Airaksinen S, Keen JE, Somero GN. 2000. Heat-shock protein expression is absent in the Artic fish *Trematomus bernacchii* (Family Nototheniidae). Journal of Experimental Biology 203:2331-2339.

Hughes T. 1994. Catastrophes, phase shifts, and large-scale degradation of a Caribbean coral reef. Science 265:1547-1551.

Hughes TP, Baird AH, Bellwod DR, Card M, Connolly SR, Folke C, Grosberg R, Hoegh-Guldberg O, Jackson JBC, Kleypas J and others. 2003. Climate change, human impacts and the resilience of coral reefs. Science 310:929-933.

Iglesias-Prieto R, Trench RK. 1994. Acclimation and adaptation to irradiance in symbiotic dinoflagellates. I. Responses of the photosynthetic unit to changes in photon flux-density. Marine Ecological Progress Series 113:163-175.

Jackson JBC. 2001. What was natural in the coastal oceans? Proceedings of the National Academy of Sciences 98:5411-5418.

Jackson JBC, Kirby MX, Berger WH, Bjorndal KA, Botsford LW, Bourque BJ, Bradbury RH, Cooke R, Erlandson J, Estes JA and others. 2001. Historical overfishing and the recent collapse of coastal ecosystems. Science 293:629-637.

Jokiel P, Coles SL. 1990. Response of Hawaiian and other Indo-Pacific Reef Corals to Elevated Temperature. Coral reefs 8:155:162.

Jokiel PL, Coles SL. 1974. Effects of heated effluent on hermatypic corals at Kahe Point, Oahu. Pacific Science 28:1-18.

Jokiel PL, Dubinsky Z, Stambler N. 1994. Results of the 1991 United States-Israel workshop, "nutrient limitation in the symbiotic association between zooxanthellae and reef-building corals". Pacific Science [PAC SCI ] Vol 48(3).

Jones RJ, Hoegh-Guldberg O, Larkum AWD, Schreiber U. 1998. Temperature-induced bleaching of corals begins with impairment of the $CO_2$ fixation mechanism in zooxanthellae. Plant, Cell and Environment 21:1219-1230.

Kinzie R, Chee GS. 1979. The effect of different Zooxanthellae on the Growth of Experimentally Re-infected Hosts. The Biological Bulletin 156:315:327.

Knowlton N, Mate JL, Guzman HM, Rowan R, Jara J. 1997. Direct evidence for reproductive isolation among the three species of the *Montastraea annularis* complex in Central America (Panama and Honduras). Marine Biology 127:705-711.

Knowlton N, Weil E, Weight LA, Guzman HM. 1992. Sibling species in *Montastraea annularis*, coral bleaching, and the coral climate record. Science 255:330-333.

Krause GH, Weis E. 1991. Chlorophyll fluorescence and photosynthesis: the basics. Annual Review of Plant Physiology and Plant Molecular Biology 42:313-349.

Kuhl M, Cohen Y, Dalsgaard T, Jorgensen BB, Revsbech NP. 1995. Microenvironment and photosynthesis of zooxanthellae in scleractinian corals studied with microsensors for O2, pH, and light. Marine Ecology Progress Series 117:159-172.

Leichter JJ, Miller SL. 1999. Predicting high-frequency upwelling: Spatial and temporal patterns of temperature anomalies on a Florida coral reef. Continental Shelf Research 19:911-928.

Lesser MP, Weis VM, Patterson MR, Jokiel PL. 1994. Effects of morphology and water motion on carbon delivery and productivity in the reef coral, *Pocillopora damicornis* (Linnaeus): Diffusion barriers, inorganic carbon limitation, and biochemical plasticity. Journal of Experimental Marine Biology and Ecology 178:153-179.

Lindquist S. 1986. The Heat Shock Response. Annual Review of Biochemistry 55:1151-1191.

Lopez JV, Kersanach R, Rehner SA, Knowlton N. 1999. Molecular determination of species boundaries in corals: Genetic analysis of the *Montastraea annularis* complex using amplified fragment length polymorphisms and a microsatellite marker. Biological Bulletin 196:80-93.

Marcus J, Thorhaug A. 1981. Pacific versus Atlantic responses of the subtropical hermatypic coral *Porites* spp. to temperature and salinity effects. Proceedings of the 4th International Coral Reef Symposium 2:15-20.

Meesters EH, Bak RPM. 1993. Effects of coral bleaching on tissue regeneration potential and colony survival. Marine Ecology Progress Series 96:189-198.

Morimoto RI. 1993. Cells in stress: Transcriptional activation of heat shock genes. Science (Washington) [SCIENCE (WASH ) ] Vol 259(5100).

Morimoto RI. 1998. Regulation of heat shock transcription response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genetics and Development 12:3788 - 3796.

Morimoto RI, Tissieres A, Geogopoulos C. 1994. Progress and perspectives on the biology of heat shock proteins and molecular chaperones. Morimoto RI, Tissieres A, Geogopoulos C, editors. 1 - 30 p.

Munk W, H., Riley GA. 1952. Absorption of nutrients by aquatic plants. Journal of Marine Research. 11(346):215-240.

Muscatine L. 1990. The role of symbiotic algae in carbon and energy flux in reef corals. In: Dubinsky Z, editor. Ecosystems of the World: Coral Reefs. Amsterdam: Elsevier. p 75-87.

Muscatine L, Porter JW. 1977. Reef corals: Mutualistic symbioses adapted to nutrient-poor environments. BioScience 27:454-460.

Nakamura T, van Woesik R. 2001. Water-flow rates and passive diffusion partially explain differential survival of corals during the 1998 bleaching event. Marine Ecology Progress Series 212:301-304.

Nakamura T, Yamasaki H, van Woesik R. 2003. Water flow facilitates recovery from bleaching in the coral *Stylophora pistillata*. Marine Ecology Progress Series 256:287-291.

Nybakken JW. 1993. Marine Biology, An Ecological Approach. Glyn D, editor. London: Harper and Collins.

Oliver JK. Recurrent seasonal bleaching and mortality of corals on the Great Barrier reef; 1985; Tahiti. p 201-206.

Pandolfi JM, Bradbury RH, Sala E, Hughes TP, Bjorndal KA, Cooke RG, McArdle D, McClenachan L, Newman MJH, Paredes G and others. 2003. Global trajectories of the long-term decline of coral reef ecosystems. Science 301:955-958.

Patterson MR. 1991. The effects of flow on polyp-level capture in an octocoral, *Alcyonium siderium*. Biological Bulletin 180:93-102.

Patterson MR, Price WS. Correlation of water motion with rates of intracolony coral bleaching; 1992; Santa Fe.

Patterson MR, Sebens KP, Olson RR. 1991. *In situ* measurements of flow effects on primary production and dark respiration in reef corals. Limnology and Oceanography 36(5):936-948.

Patterson MR, Sebens PK. 1989. Forced convection modulates gas exchange in cnidarians. Proceedings of the National Academy of Sciences 86:8833-8836.

Robbart M, L., Peckol P, Scordilis S, P., Curran H, A., Brown-Saracino J. 2004. Population recovery and differential heat shock protein expression for the corals Agaricia agaricites and A. tenuifolia in Belize. Marine Ecology Progress Series 283:151-160.

Robbart ML. 2002. Population dynamics and differential heat shock protein expression in two Caribbean scleractinian corals, Agarica Agaricites and A, Tenuifolia [Masters]: Smith College. 98 p.

Rosenberg E, Loya Y. 2004. The Adaptive Hypothesis of Coral Bleaching. In: Rosenberg E, Loya Y, editors. Coral Health and Disease. New York: Springer-Verlag.

Rowan R, Knowlton N. 1995. Intraspecific diversity and ecological zonation in coral-algal symbiosis. Proceedings of the National Academy of Sciences 92:2850-2853.

Rowan R, Knowlton N, Baker A, Jara J. 1997. Landscape ecology of algal symbionts creates variation in episodes of coral bleaching. Nature (London) 388(6639):265-269.

Rowan R, Powers DA. 1991. A Molecular Genetic Classification of Zooxanthellae and the Evolution of Animal-agal Symbiosis. Science 251:1348-1351.

Saxby T, Dennison WC, Hoegh-Guldberg O. 2003. Photosynthetic responses of the coral *Montipora digitata* to cold temperature stress. Marine Ecology Progress Series 248:85-97.

Schlichter D, Liebezeit G. 1991. The natural release of amino acids from the symbiotic coral Heteroxenia fuscescens (Ehrb.) as a function of photosynthesis. Journal of Experimental Marine Biology and Ecology Vol 150(1).

Schrieber U, Bilger W, Neubauer C. 1994. Chlorophyll fluorescence as a nonintrusive indicator for rapid assessment of *in vivo* photosynthesis. In: Schulze ED, Caldwell MM, editors. Ecophysiology of Photosynthesis. Berlin: Springer. p 49-70.

Schrieber U, Schliwa U, Bilger W. 1986. Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new modulation fluorometer. Photosynthesis Research 10:51-62.

Sebens KP, Grace S, Helmuth BST, Maney E, Miles J. 1998. Water flow and prey capture by three scleractinian corals, *Madracis mirabilis*, *Montastrea cavernosa* and *Porites porites* in a field enclosure. Marine Biology 131:347-360.

Sebens KP, Helmuth BST, Carrington E, Agius B. 2003. Effects of water flow on growth and energetics of the scleractinian coral *Agaricia teniufolia* in Belize. Coral Reefs 22:35-47.

Sebens KP, Witting J, Helmuth BST. 1997. Effects of water flow and branch spacing on particle capture by the reef coral *Madracis mirabilis* (Duchassaing and Michelotti). Jounal of Experimental Marine Biology and Ecology 211:1-28.

Sharp VA, Brown BE, Miller D. 1997. Heat shock protein (HSP 70) expression in the tropical reef coral *Goniopora djiboutiensis*. Journal of Thermal Biology 22(1):11-19.

Somero GN, DeVries AL. 1967. Temperature tolerance of some Antarctic fishes. Science 156:257-258.

Szmant AM, Gassman NJ. 1990. The effects of prolonged "bleaching" on the tissue biomass and reproduction of the reef coral *Monastrea annularis*. Coral Reefs 8:217-224.

Thomas F, Atkinson M. 1997. Ammonium uptake by coral reefs: Effects of water velocity and surface roughness on mass transfer. Limnology and Oceanography 42:81-87.

Toller WW, Rowan R, Knowlton N. 2001. Zooxanthellae of the *Montastrea annularis* species complex: Patterns of distribution of four taxa of *Symbiodinium* on different reefs and across depths. Biological Bulletin 201:348-359.

Tomanek L, Somero GN. 2000. Time course and magnitude of synthesis of heat-shock proteins in congeneric marine snails (genus *Tegula*) from different tidal heights. Physiological and Biochemical Zoology 73(2):249-256.

Trench RK. 1993. Microalgal-invertebrate symbioses: A review. Endocytobiosis and Cell Research 9:135-175.

Van Veghel MLJ, Bak RPM. 1993. Intraspecific variation of a dominant Caribbean reef building coral, *Montastrea annularis*: Genetic, behavioral and morphometric aspects. Marine Ecology Progress Series 92:255-265.

Vaughan TW. 1914. Reef corals of the Bahamas and of Southern Florida. Carnegie Institute Washington Yrbk 13:222-226.

Vogel S. 1994. Life in Moving Fluids. Princeton: Princeton University Press. 467 p.

Ware JR, Fautin DG, Buddemeier RW. 1996. Patterns of Coral Bleaching: Modeling the Adaptive Bleaching Hypothesis.

Warner ME, Chilcoat GC, McFarland FK, Fitt WK. 2002. Seasonal fluctuations in the photosynthetic capacity of photosystem II in symbiotic dinoflagellates in the Caribbean reef-building coral *Montastrea*. Marine Biology 141:31-38.

Warner ME, Fitt WK, Schmidt GW. 1996. The effects of elevated temperature on the photosynthetic efficiency of zooxanthellae *in hospite* from four different species of reef corals: a novel approach. Plant, Cell and Environment 19:291-299.

Warner ME, Fitt WK, Schmidt GW. 1999. Damage to photosystem II in symbiotic dinoflagellates: A determinant of coral bleaching. Proceedings of the National Academy of Sciences 96:8007-8012.

Weil E, Knowlton N. 1994. A multi-character analysis of the Caribbean coral *Montastraea annularis* (Ellis and Solander, 1786) and its two sibling species, *M. faveolata* (Ellis and Solander, 1786) and *M. franksi* (Gregory, 1895). Bulletin of Marine Science 55:151-175.

Wells JW. 1956. Scleractinia. In: Moore RC, editor. Treatise on Invertebrate Paleontology. Lawrence: University of Kansas Press. p F328-F444.

West JM, Salm RV. 2003. Resistance and resilience to coral bleaching: Implications for coral reef conservation and management. Conservation Biology 17(4):956-967.

Wilkinson C. 1996. Global change and coral reef: impacts on reefs, economies and human cultures. Global Change Biology 2:547-558.

Wilkinson C. 2000. Status of coral reefs of the world. Australia: Global Coral Reef Monitoring Network.

Williams EH, Jr., Bunkley-Williams L. 1990. The world-wide coral reef bleaching cycle and related sources of coral mortality. Atoll Research Bulletin 335(1-71).

Yonge CM, Nicholls AG. 1931a. Studies on the physiology of corals. IV. The effect of starvation in light and in darkness on the relationship between corals and zooxanthellae. Scientific Report of the Great Barrier Reef Expedition 1928-29 1:178-210.

Yonge CM, Nicholls AG. 1931b. Studies on the physiology of corals. IV. The structure, distribution, and physiology of the zooxanthellae. Scientific Report of the Great Barrier Reef Expedition 1928-29 1:135-176.

# CHAPTER 1

# WATER FLOW INFLUENCES THE SPATIOTEMPORAL DISTRIBUTION OF PHOTOSYNTHETIC EFFICIENCY WITHIN COLONIES OF THE CORAL *MONTASTREA ANNULARIS* (ELLIS AND SOLANDER, 1786): IMPLICATIONS FOR CORAL BLEACHING

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## ABSTRACT

Recent studies have determined that water-flow may perform a number of roles during and following episodes of coral reef bleaching. Increased water-flow has the potential to improve the capacity of some scleractinian corals to cope with the high concentrations of oxygen in the boundary layer that are photosynthetically derived, whereas bleaching is often at its highest in areas where water-flow is restricted. Conversely, water motion has been shown to increase the metabolic rate both for photosynthesis and respiration and such flow-modulated metabolism may result in asymmetric bleaching patterns within a coral colony. To broaden these earlier studies and to further examine the effects of flow on photosynthetic efficiency, we investigated whether corals would exhibit a spatially asymmetric distribution of photosynthetic efficiency related to velocity gradients (degree of mixing) across a coral colony.

Experimental manipulations were conducted from the NOAA underwater habitat Aquarius on colonies of *Montastrea annularis* with controlled exposures to increased flow (*ca.* 40 cm $s^{-1}$) 1 cm above coral surface and increased temperature (*ca.* 1.5 - 2 °C above ambient) using *in situ* flow chambers. Photosynthetic efficiency was measured as a light-adapted quantum yield ($F_v'/F_m'$), using a pulse amplitude modulated (PAM) fluorometer to examine the short term – (daily) and medium term –(9 days) response of patches of polyps within coral colonies. *Montastrea annularis* colonies developed and

sustained significant spatially asymmetric patterns of photosynthetic yield across the entire coral surface, with the upstream side of the colonies exhibiting reduced quantum yield. At the same time, the upstream side developed and sustained an increased regulation within the synthesis of stress proteins (*cf.* Chapter 3). The mechanism producing this pattern is unclear; we speculate that increased flow may lead to an up-regulation of photosynthesis by the entire colony through bicarbonate delivery accentuated by the $Q_{10}$ effect. Local down-regulation of the photosynthetic response (decreased quantum yield) might then occur to keep tissue oxygen concentrations within tolerable limits.

# INTRODUCTION

There are several natural and anthropogenic triggers of coral stress: *e.g.*, thermal extremes (Brown 1997; Brown and others 2002b; Buddemeier and Fautin 1993; Jones and others 1998; Saxby and others 2003), light saturation (Banaszak and Trench 1995; Brown 1997; Jokiel and Coles 1974), bacterial infection (Ben-Haim and others 1999; Ben-Haim and others 2003; Hughes 1994; Richardson and others 1998), sedimentation (Brown 1997; Cole 2003), and high irradiance including UV (Gleason and Wellington 1993), all of which are associated with a host of deleterious impacts on corals (Brown 1997; Fitt and Warner 1995; West and Salm 2003). Coral stress can often occur when the regular operating limits of the aforementioned triggers drift outside the corals' narrow range of optimum environmental conditions. In many cases these environmental excursions can initiate the breakdown of the coral's metabolic functions and culminate in the expulsion of the coral's algal symbiont and/or degradation of the symbiont's photosynthetic pigments (Brown 1997; Brown and others 2002a; Downs and others 2002). Following the loss of symbiont and/or pigment the effected coral colonies or reef can often appear white or bleached (Brown 1997; Glynn 1993; Hoegh-Guldberg 1999). Bleaching events are often accompanied by but not restricted to a reduction in reproductive effort, an increased susceptibility to disease, and a loss of functioning coral, including mortality within a range of temporal and spatial scales (Brown 1997; Brown and others 2002a).

*Water Flow and Coral Stress*

There are a number of mechanisms through which enhanced water flow-rates could modulate coral bleaching. Enhanced water-flow stimulates photosynthesis within a coral colony at a local scale through the reduction of various boundary layer thicknesses (Patterson and others 1991; Shashar and others 1996). High irradiance and increased flow are proposed as a mechanism that may limit the supply of dissolved inorganic carbon (DIC) through the increased translocation of carbon rich photosynthate (Lesser and others 1994). High flow rates and thinner boundary layers conspire to reduce the build-up of oxygen during periods of elevated temperatures, as oxygen can become supersaturating during periods of reduced flow velocities (Finelli and others 2006; Gardella and Edmunds 1999; Shashar and others 1993). Higher water-flow velocities, however, may only provide a short-term solution due to the metabolic cost of up-regulating the synthesis of stress proteins, *e.g.*, heat shock protein 70 (*hsp*70), required to cope with various stressors, *e.g.*, coincident higher temperatures (Carpenter and Patterson, unpublished data).

Although many of the known environmental triggers for bleaching have been studied, a proposed model for the cellular mechanisms of coral bleaching posits *oxidative stress* through increased concentrations of toxic (photo-derived) oxygen and/or oxygen radicals (Downs and others 2002; Finelli and others 2006; Lesser 1997). Oxygen and its derivatives are transferred between coral and the surrounding water via diffusion and this diffusive flux is inversely proportional to the boundary layer thickness and directly proportional to the environmental (freestream) concentration. Since flow directly

modulates boundary layer thickness and alters the availability of oxygen in the freestream, flow may also modulate the metabolic rates of coral at a range of spatial scales from single polyps and coral colonies to sections of entire coral reefs (Gardella and Edmunds 1999; Lesser and others 1994; Patterson 1991; Sebens and others 2003; Shashar and others 1993; Shashar and others 1996; Thomas and Atkinson 1997).

The conceptual model presented by Shashar et al. (1996) summarizes the current understanding of mass transfer (Finelli and others 2006). At spatial scales of *ca.* 1 - 100 m *e.g.*, such as whole reef tracts, Benthic Boundary Layers (BBL), are formed as ocean currents interact with the reef, supplying dissolved nutrients and setting the physical context for smaller-scale processes. At scales of *ca.* 1 - 100 cm (*e.g.*, individual coral colonies), organisms are encapsulated within Momentum Boundary Layers (MBL) created as currents flow over the coral surface. Mass transfer is controlled by the MBL modulating the Diffusive Boundary Layers (DBL) through shear stress over the coral surface (Finelli and others 2006; Gardella and Edmunds 2001; Patterson 1992; Shashar and others 1996). The DBL is in the region closest to the surface of the coral, and it is here that mass transport is carried out by molecular diffusion as opposed to convective mass transfer. Therefore the thickness of the DBL determines the rate of mass transfer to and from the coral, which can vary with the shear stress within the MBL. Within true environmental water-flows, the DBL is usually less than a few millimeters in thickness (Finelli and others 2006; Gardella and Edmunds 1999; Patterson 1992; Shashar and others 1996). Flow-modulated metabolism has already been demonstrated within the respiration of octocorals and sea anemones (Patterson and Sebens 1989) and

photosynthesis and respiration in *M. annularis* (Patterson and others 1991). Patterson and Sebens (1989) cautioned that when comparing measurements made in an enclosed chamber to those in the field, chamber-induced flow artifacts (*e.g.*, blockage effects) required consideration. Some pervasive problems associated with these experimental designs are, the acceleration of flow between the organism and chamber wall and the unidirectional nature of the chamber flow, compared to the bi-directional nature of many shallower habitats (Patterson and Sebens 1989). These observations apply to this study and were accounted for by a size restriction on the coral treatments (to maximize the free-space between coral and chamber wall) in tandem with video analyses to resolve water-flow behavior at the coral-polyp level. Notwithstanding these potentially confounding artifacts, Patterson and Sebens' (1989) findings were in qualitative agreement with a range of previous studies. In all cases, enhanced flow speed increased the rate of gas exchange, and this process occurred at the coral-polyp level (Patterson and Sebens 1989). The question, does the rate of gas exchange scale with increasing flow speed at the reef or landscape level, still requires attention.

This study was specifically designed to address the role of water-flow on coral bleaching within the scleractinian coral *M. annularis* using *in situ* heated flow chambers, measuring photosynthetic yield on small patches of polyps within a colony. We addressed the central question: Is coral bleaching, as measured by photosynthetic efficiency within a colony, modulated by water-flow? The primary focus of this study was: (i) to establish and maintain six individual coral colonies in self-contained coral

microcosms during two ten-day saturation missions, (ii) to systematically collected time series fluorescence emission data from four spatially distinct coral sectors, and (iii) resolve any spatiotemporal effect that an enhanced water-flow may impose on photosynthetic efficiency, across the up and downstream surfaces of individual coral colonies relative to flow. Field observations during bleaching events in Jamaica (1990), Florida (1991) and subsequent flume experiments showed a preliminary pattern of upstream/downstream asymmetry in bleaching with colonies of *M. annularis* and *M. cavernosa* (Patterson and Price 1992).

During bleaching events, corals exposed to well-mixed flow regimes *e.g.*, due to local bathymetry, or reduced temperatures *e.g.*, through bathymetry or depth, may acquire an enhanced *metabolic state* and be better placed maintain normal cellular functions such as photosynthesis. We speculate, that corals located in other areas on the same reef may experience micro-environmental conditions too intense to maintain photosynthesis and flow-stress may then manifest as an asymmetric pattern of photosynthetic efficiency across the coral colony relative to flow, and similar to that of stress protein synthesis (*cf.* Chapter 3).

# MATERIALS AND METHODS

## *Study Location and Underwater Laboratory*

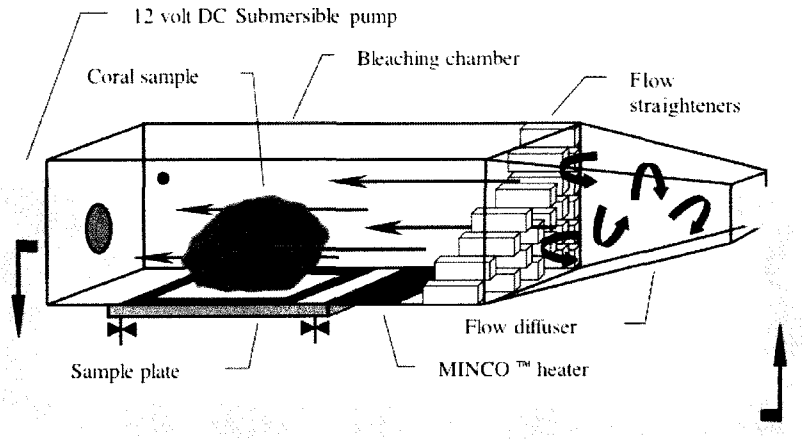
The *in situ* studies described here were conducted at the NOAA Aquarius underwater habitat located within the Florida Keys National Marine Sanctuary at 24° 57.230' N, 80° 27.223' W, during two (ten day) saturation missions in November 2002 and July 2003. On a daily basis several sampling methods were carried out by four saturated divers. The nearby reef is characterized by spur and groove formations to depths of 40 m and is the focus of a wide range of research.

## *Coral Bleaching Flow Chambers*

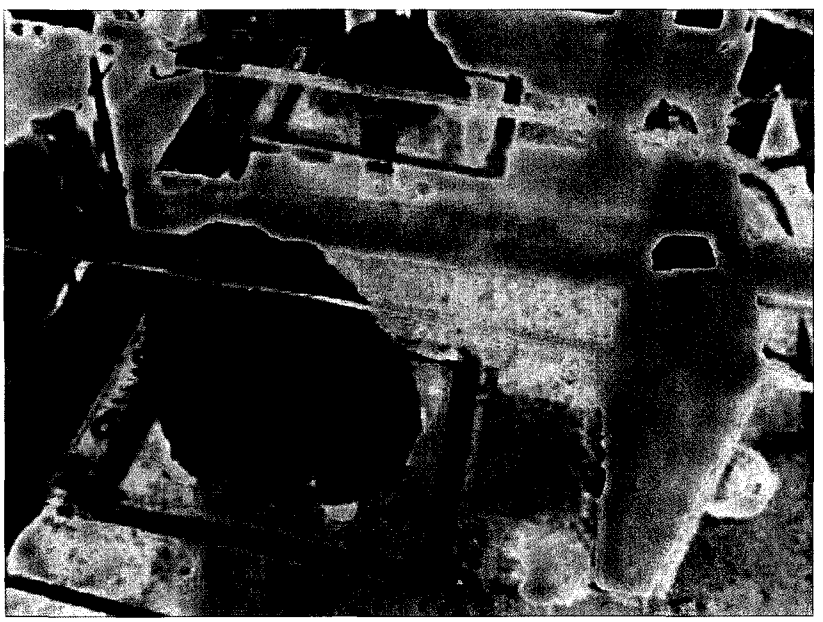
Six (7.1 L) uni-directional re-circulating coral bleaching flow chambers fabricated from 6.4 mm Plexiglas were modified from the original design (Patterson and others 1991) (Figure 1 A.). Water-flow enters the main body of the chamber via an upstream diffusing cone at speeds of *ca.* 8 - 10 cm $s^{-1}$ (Patterson and others 1991). The angle of the cone was chosen to minimize flow separation. The flow passes through a bank of rigid plastic flow straightners (i.d., 5 mm; length, 80 mm) and passes over the coral sample which is held in position on a removable Plexiglas plate with an inert sealing compound (Mortite) (Patterson and others 1991) (Figure 1 B.).

**Figure 1. A.** Coral bleaching flow chamber design. **B.** Screenshot of two chambers with samples *in situ*. Minco Thermofoils heaters with embedded RTD sensor were located upstream of all six coral samples and in conjunction with heating from turbulence decay, provided a controlled temperature increase (1.5 - 2.0° C) above the ambient temperatures on the reef. Each heater was connected to a separate Minco PID temperature controller inside the habitat. The sample plate allowed for daily collection of coral polyps for heat shock protein analysis (*cf.* Chapter 3) and QY measurements using a Diving PAM fluorometer.

A




B



This plate allowed for the daily removal of the coral colony, reducing the stress from over handling.

Aquarius supplied electrical power to the pumps and heaters. Re-circulating flow within each chamber was provided by a 13.8 VDC, 7 Amp 1500 GPH (6300 L $hr^{-1}$) Rule bilge pumps mounted externally on the downstream side of the chambers. The water-flow was returned to the upstream diffusing cone via a system of external PVC tubing (o.d. 35 mm; i.d. 28 mm). Minco Thermofoil heaters (ASI 5902 R9.50 PFB) were fixed to the floors of all six bleaching chambers upstream of the coral samples. An integrated platinum resistance temperature device (RTD) combined with the natural decay of the turbulence of the flow passing through the chamber plumbing, maintained the water temperature within each chamber at *ca.* 1.5 - 2 °C above ambient. The water temperature of each chamber was monitored and controlled from inside Aquarius, via a Minco (CT16A2020) PID temperature controller and associated Crydom 20 Amp (AC1009) solid-state relay.

Accuracy of the chamber temperature controllers was confirmed daily with a hand-held alcohol thermometer (resolution of 0.1 °C), through several small access ports along the length of the chambers. Given the high flow output of the Rule pumps and vigorous mixing, no temperature gradient was detected along the length of the chambers. During the course of the experiment, ambient temperatures fluctuated on the order of 1.5° C and these fluctuations were reflected, although delayed, in the record of chamber temperatures. During the 2002 field season, chamber heaters partially failed midway

through the mission, and thus the heating effect was diminished by *ca.* 1° C for some chambers during the last 3 days. All chambers, however, consistently showed readings *ca.* 1.0 - 1.5° C above ambient during the entire 2002 deployment, in part due to turbulence decay. While saturated, aquanauts have few restrictions on their dive-times and were able to monitor, repair, sample, photograph and maintain any chamber and coral sample independently of the other experimental set-ups.

***Coral samples***

The subject species was the hermatypic scleractinian coral *M. annularis*, the predominant reef-building species throughout the Caribbean and the Florida Keys. Prior to the 2002 fieldwork, collection permits were obtained from the Florida Keys National Marine Sanctuary to harvest 12 (*ca.* 150 mm x 150 mm x 150 mm) mounding and flat plate morphologies of the hard coral *M. annularis*, within 1.6 km of Aquarius. A minimum cut-off depth of *ca.* 18 m ensured the collection of the correct morphotype of interest within the *Montastrea* species-complex *cf.,* (Knowlton and others 1997; Knowlton and others 1992; Lopez and others 1999). Divers randomly identified potential samples using waterproof color-image guides. Before the removal of any sample, checks were made with a 120 x 120 mm test-frame to determine that the sample colony would physically fit into the chamber. The coral sample and surrounding area were then photographed and prevailing water-flow recorded via compass bearings so that colonies could be oriented in the chamber with the dominant flow direction similar to that experienced in the field. Coral samples were cleaved off the reef substrate with a masonry hammer and a 130 mm blade-width steel chisel. Samples were transferred to

the surface in individual covered containers to limit light exposure prior to the diver fully surfacing. During transportation to the habitat, the water temperature was monitored and regulated by keeping samples out of the sun in an insulated cooler. The coral samples were re-positioned adjacent to Aquarius on the nearby sand plain at a depth of 22 m (which was within a few meters of the collection depths), and allowed to acclimate for three days prior to the start of the science mission. Corals were given an identification number that was retained for both years. Between and following the 2002 and 2003 saturation missions, all coral samples were tagged and reattached to the reef adjacent to Aquarius with Z-spar marine epoxy, photographed *in situ* and the location mapped. Thus the original coral samples were used for the 2003 season.

***Photosynthetic Performance and the Collection of Fluorescence Emission data***

Undisturbed corals located in conditions of ambient flow and temperature, were used to provide a base line for quantum yield (QY), and are discussed below. Daily, three coral colonies of the same morphotype and similar size were measured for photosynthetic efficiency throughout 2002 and 2003.

Pulse amplitude modulated and the fast repetition rate (FRR) fluorometers (Falkowski and others 2004; Gorbunov and others 2000; Schreiber 2004) rely on the relationship between chlorophyll fluorescence and the rate of electron transfer within Photosystem II (PSII). For an overview of the development and application of chlorophyll fluorescence in the marine ecosystem, Pulse-Amplitude-Modulated (PAM)

fluorometry and saturation pulse method, see the following sources: (Falkowski and others 2004; Jones and others 1999; Schreiber 2004; Warner and others 2002).

The PAM fluorometer initially measures chlorophyll fluorescence under ambient light conditions ($F_o'$) and then subjects the coral to a saturating pulse of actinic light and re-measures the fluorescence ($F_m'$). Due to the logistics of the saturation mission and chamber deployment, we were unable to collect dark-adapted fluorescence measurements. The analysis of light-adapted coral readings thus has to account for the effect of prior exposure to ambient light ($F_o'$) experienced by PSII, prior to the fluorescence measurements. To this end, and on the assumption that each sample coral on average experienced the same level of prior irradiance, fluorescence analysis focused on the *relative changes* of the polyp-by-polyp measurements within a coral sample for the duration of the experiment. All chlorophyll fluorescence measurements should be regarded as light adapted (Falkowski and others 2004).

The quantum yield (QY) is computed from two fluorescence signals as:

$(F_m' - F_o') = F_v'$

$(F_v' / F_m') = QY$, Quantum yield

where:

$F_m'$ = Fluorescence max light adapted

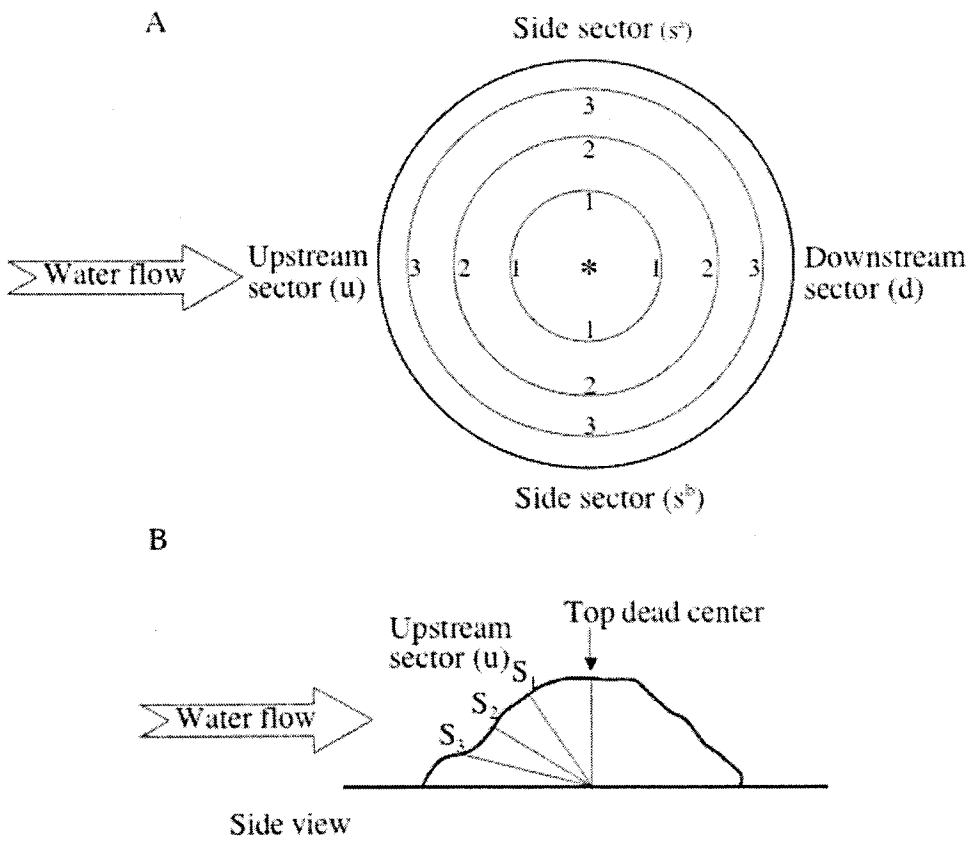
$F_o'$ = Fluorescence initial light adapted

$F_v'$ = Fluorescence variable light adapted

Quantum yield is proportional to the amount of incident radiation that can be utilized to facilitate photosynthesis. Elevated values of QY correspond to an increased efficiency in the use of incident radiation and higher rates of photosynthesis (Finelli and others 2006; Schreiber 2004; Schrieber and others 1994).

*In situ* fluorescence emissions were measured from the coral samples using a Diving-PAM fluorometer (Walz, GmbH, Effeltrich, Germany). Once a day throughout the saturation mission, divers systematically removed the corals from the chambers (without touching them) using the sample plate collected fluorescence emission data from four spatially distinct coral sectors: one upstream, two side positions, and one downstream referenced as: *up* - *side*$^{a}$ - *side*$^{b}$ - *down* (u-s$^{a}$-s$^{b}$-d) (Figure 2 A.). Divers would locate the top dead center of the coral, the move the fiber optic probe of the Diving-PAM down one quarter of the distance along the downstream sector and take a reading with the probe, 5 mm above the coral surface, move another quarter of the way down from the top, and so on until three separate and equally spaced measurements were obtained (Figure 2 B). This procedure was repeated for the two side and downstream sectors. The diameter of the Diving-PAM probe is approximately that of four polyps; thus the same patch of polyps was measured each day. The Diving-PAM also simultaneously recorded, Photosynthetically Active Radiation (PAR) irradiance, using a cosine sensor.

**Figure 2. A.** Top view of the location of the 13 data points collected daily from each coral colony using a Diving PAM fluorometer. **B.** Side view of the sampling locations and nomenclature.



For comparison, divers collected PAM data from > 100 undisturbed native corals including the *Montastrea* species complex at different depths and times of day. As part of a larger study (Chap. 3) carried out during the same daily PAM sampling regime, a number of polyp samples were initially harvested from all the experimental coral colonies to record the baseline level of constitutive stress protein. Thereafter and for the remainder of the experiment, all coral colonies were sampled each day for a later study on stress protein up-regulation. Underwater digital photography was used to record the daily loss of pigmentation across the corals surface.

***Flow Visualization***

A visual record of the water-flow, over each coral sample was made by filming hydrated brine shrimp cysts passing over the coral in the chamber. The cysts were illuminated with a modified (25 W) hand-held divers halogen lamp that cast a collimated beam of light (*ca.* 80 mm x 3 mm) perpendicular to the water-flow over the coral sample. The video was recorded using a Sony Hi 8 mm analog camcorder in an underwater housing. The Hi 8 mm videotape was digitized using iMovie on an Apple G4 using a Sony analog Hi 8 mm control deck. For each of the sectors (n = 4) (u, $s^a$, $s^b$, d) and at three heights (0.3, 0.6, 1.0 cm) above the coral polyps, individual shrimp cysts (n = 3) were selected and tracked for five frames (1/6 s), then averaged.

*Reynolds numbers*

To further characterize the nature of the flow régime over the colonies, we used the Reynolds number (Re), calculated from flow speed ($u$), colony head height ($W$), and momentum diffusivity (kinematic viscosity) of the fluid ($v$):

$$Re = uW/v.$$

The Re is the ratio of inertial to viscous forces that impinge on parcels of moving fluid, and therefore serves as an index of the gross characteristic of the flow over each coral colony (Patterson and others 1991; Schlichting and others 2000). Laminar flow (Re = < 2300) occurs when a fluid flows in organized parallel layers with little or no disruption between the layers and is characterized by diffusive transport mechanisms within the boundary layer (Vogel 1996). Turbulent flow (Re = > 2300) is chaotic, disorganized and characterized by lots of cross-stream transport.

# RESULTS

## *Statistical analysis*

The 2002 and 2003 QY data sets were analyzed through a repeated measure analysis of covariance (ANCOVA) (Kutner and others 2005) to determine if significant differences existed between and within areas of high and low water-flow during the two (8 day – 2002; 9 day - 2003) treatments. These analyses were performed using QY as the response variable, the individual colonies (n = 5), sector (n = 4) and level (n = 3) (within each sector) as factors, and day and Photosynthetic Active Radiation (PAR) as the covariates. All fluorescence yield data, which are ratios, were first arcsine transformed to generate a normal distribution for analysis and then back-transformed for graphing (Kutner and others 2005).

The 2002 flow data sets were analyzed using a 3-way analysis of variance (ANOVA) to determine if significant differences were present between and within discrete sectors within the coral (u, $s^a$, $s^b$, d), referenced to flow and specific heights (0.3, 0.6, 1.0 cm) above the coral during the (8 day) treatment in 2002. These analyses were performed using the flow speed data calculated from the flow-visualization video as the response variable, sector (n = 2) and height (n = 3) as factors, and coral colony (n = 6) as a random factor. All statistical analyses were performed using MINITAB (version 14) and graphing was carried out using GraphPad Prism (version 4).

For a summary of ANCOVA and ANOVA results see Table 1, and for an example of a typical ANCOVA output (2003) see Table 2.

**Table 1.** Summary of ANCOVA results for quantum yield (QY) measurements over both years. Note that the effect of 'coral colony' was significant in both years and all transects. The effect of the factor 'sector' was significant in both years except for transect us$^{b}$d in 2002. In the 3 wild corals, 'colony' was significant in both years but 'sector' was not. Chamber temperature had a significant effect in 2002, but not 2003, when better temperature regulation was achieved. The ANOVA results for the flow speeds in 2002 reported 'coral colony', 'height' (of measured flow), and 'sector' to all be significant, but the interaction term 'sector*height' was not. All statistical analyses were performed using MINITAB (version 14).

| Parameter | **Transect - us$^{a}$s$^{b}$d** | | **Transect - us$^{a}$d** | | **Transect - us$^{b}$d** | | **Wild corals** | |
|---|---|---|---|---|---|---|---|---|
| | 2002 | 2003 | 2002 | 2003 | 2002 | 2003 | 2002 | 2003 |
| | | | **Quantum yield P values** | | | | | |
| *Coral colony* | *< 0.0005* | *< 0.0005* | *< 0.0005* | *0.006* | *< 0.0005* | *0.018* | *< 0.0005* | *< 0.0005* |
| Sector | *0.015* | *0.011* | *0.021* | *0.013* | n.s. | *0.003* | n.s. | n.s. |
| Level | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n/a | n/a |
| Sector*Level | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n/a | n/a |
| | | | **Temperature P values** | | | | | |
| *Chamber* | *0.013* | n.s. | *0.025* | n.s. | *0.015* | n.s. | n/a | n/a |
| | | | **Flow speed P values** | | | | | |
| *Coral colony* | *< 0.0005* | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| Height | *< 0.0005* | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| Sector | *< 0.0005* | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| Sector*Height | n.s. | n/a | n/a | n/a | n/a | n/a | n/a | n/a |

**Table 2.** General linear model; Quantum yield (QY), versus 'colony', 'sector' and 'level'. Summary (example) of ANCOVA output for QY measurements (2003), including the identification of key statistical elements. All statistical analyses were performed using MINITAB (version 14)

| Factor | Type | Levels | Values |
|---|---|---|---|
| Colony | Fixed | 5 | 1, 2, 3, 4, 5, 6 |
| Sectors | Fixed | 4 | u, $s^a$, $s^b$, d |
| Level | Fixed | 3 | 1, 2, 3 |

**Analysis of Variance for Arcsine QY using Adjusted SS for Tests**

| Source | DF | F | P value |
|---|---|---|---|
| Day | 1 | 49.92 | *< 0.0005* |
| PAR | 2 | 210.33 | *< 0.0005* |
| Colony | 4 | 6.15 | *< 0.0005* |
| Sectors | 3 | 4.35 | *0.005* |
| Level | 2 | 2.14 | *0.119* |
| Position*Level | 6 | 1.08 | *0.371* |

| Term | Coef | SE Coef | T | P value |
|---|---|---|---|---|
| Constant | 0.833815 | 0.003226 | 258.50 | *< 0.0005* |
| Day | 0.003738 | 0.000529 | 7.07 | *< 0.0005* |
| PAR | -0.000887 | 0.000061 | -14.50 | *< 0.0005* |

***Covariate Interpretation (up vs. downstream) and Analysis of Photosynthetic Efficiency* (2002 & 2003)**

The effects of the covariates 'PAR' and 'day' were significant, with 'PAR' (2002) negatively affecting photosynthetic efficiency ($p < 0.0005$, - 0.000075 QY units per $\mu$moles photons $m^{-2}$ $day^{-1}$) and time (day) also showing a negative affect ($p = 0.042$, - 0.0062 QY units per day). For 2003, the effect of the covariates 'PAR' and 'day' were also significant, with 'PAR' (2003) negatively affecting photosynthetic efficiency ($p < 0.0005$, - 0.00089 QY units $\mu$moles photons $m^{-2}$ $day^{-1}$) and time (day) showing a positive effect of ($p < 0.0005$, 0.0037 QY units per day). The difference between the coefficients, however, was less than the standard error of each coefficient, and therefore not significant.

The factor, 'level', and a 'position*level' interaction term were included within all QY analyses, neither of which was statistically significant in either year. A 'sector*level' interaction term was included in all chamber velocity profile analyses but was also not statistically significant. For flow velocities at specific locations and heights above coral treatments see Table 1.

***Quantum Yield (2002 & 2003)***

The quantum yield, ($F_v'/F_m'$) of PS II was collectively analyzed within the four sectors of the treated colonies (n = 5), during both (8 & 9 day) treatments periods in 2002 and 2003, down three along-flow transects within each colony, that is u-$s^a$-$s^b$-d, u-$s^a$-d and u-$s^b$-d. Three factors were analyzed across the corals surface: 'colony', 'sector' and

'level' (Figure 3). A repeated measure ANCOVA (Kutner and others 2005) resolved significant differences within the QY, expressed in asymmetrical patterns across the treated corals over two field seasons and at different times of the year, that is November 2002 and July 2003.

For the along-flow longitudinal transect u-$s^a$-$s^b$-d (Figure 2 A) (2002 & 2003), the factor 'colony' was significant in both years ($p < 0.0005$), for the transect u-$s^a$-d, 'colony' was significant in 2002 ($p < 0.0005$) and 2003 ($p = 0.006$) and for the transect u-$s^b$-d, 'colony' was significant in 2002 ($p < 0.0005$) and 2003 ($p = 0.018$).

For the along-flow longitudinal transect u-$s^a$-$s^b$-d (Figure 2 A), the factor 'sector' was significant in 2002 ($p = 0.015$) and 2003 ($p = 0.011$), for the transect u-$s^a$-d, 'sector' was significant in 2002 ($p = 0.021$) and 2003 ($p = 0.013$). For transect u-$s^b$-d, 'sector' was not significant in 2002 but was significant in 2003 ($p = 0.003$).

***In situ Corals (A B C) (2002 & 2003)***

In addition to the manipulated treatments, the QY ($F_v'/F_m'$), was randomly collected and then collectively analyzed for a set of randomly selected *in situ M. annularis* colonies (n = 3) located in ambient flow and temperature conditions at the same depth near the Aquarius habitat. The factor 'colony' was significant in both years ($p < 0.0005$), but 'sectors' were not (Figure 4).

**Figure 3.** Quantum yield (QY) (dimensionless), in light adapted colonies, in each of the two sample years 2002 and 2003, by position (u - upstream, $s^a$, $s^b$ - sides a and b, respectively, and d - downstream), and along the water-flow direction over the surface of the experimental colonies. *Error bars* are standard errors of the mean as reported in the analysis of covariance and means represent the average within the separately pooled sectors (n = 4) during both field seasons (8 days – 2002; 9 days – 2003) where u = upstream, $s^{a,b}$ are sides a and b, respectively, and d = downstream sectors. Note the difference of QY between the upstream and downstream sectors. Data were arcsine transformed prior to computations and then back-transformed for graphical portrayal.

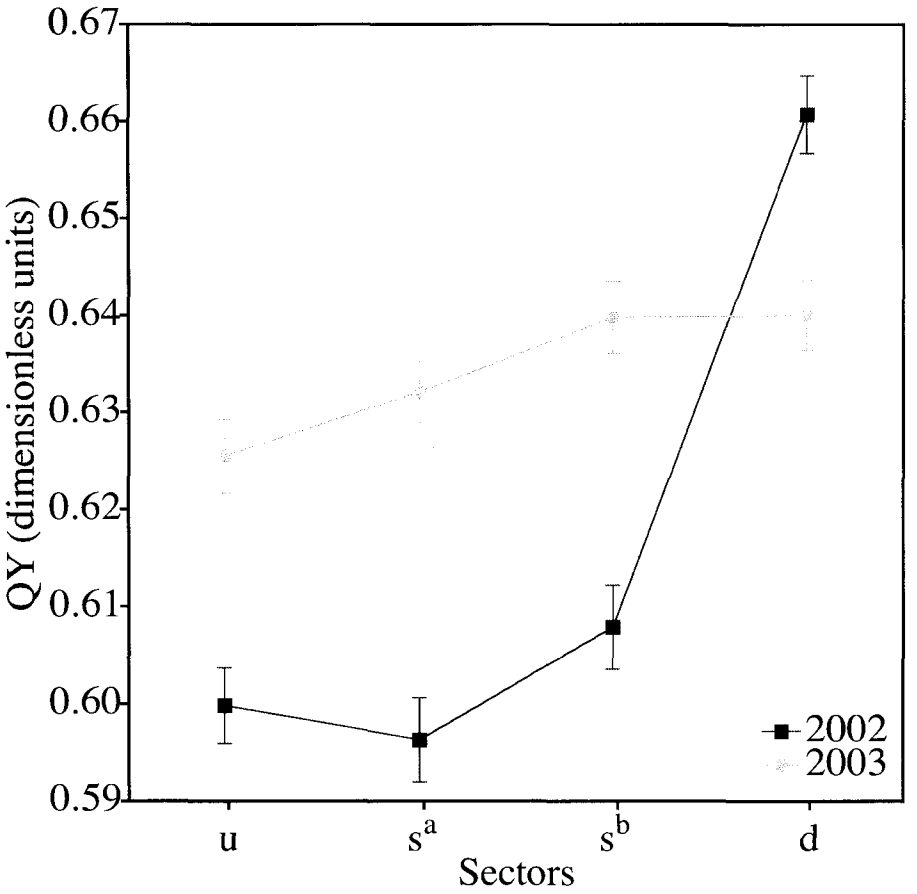
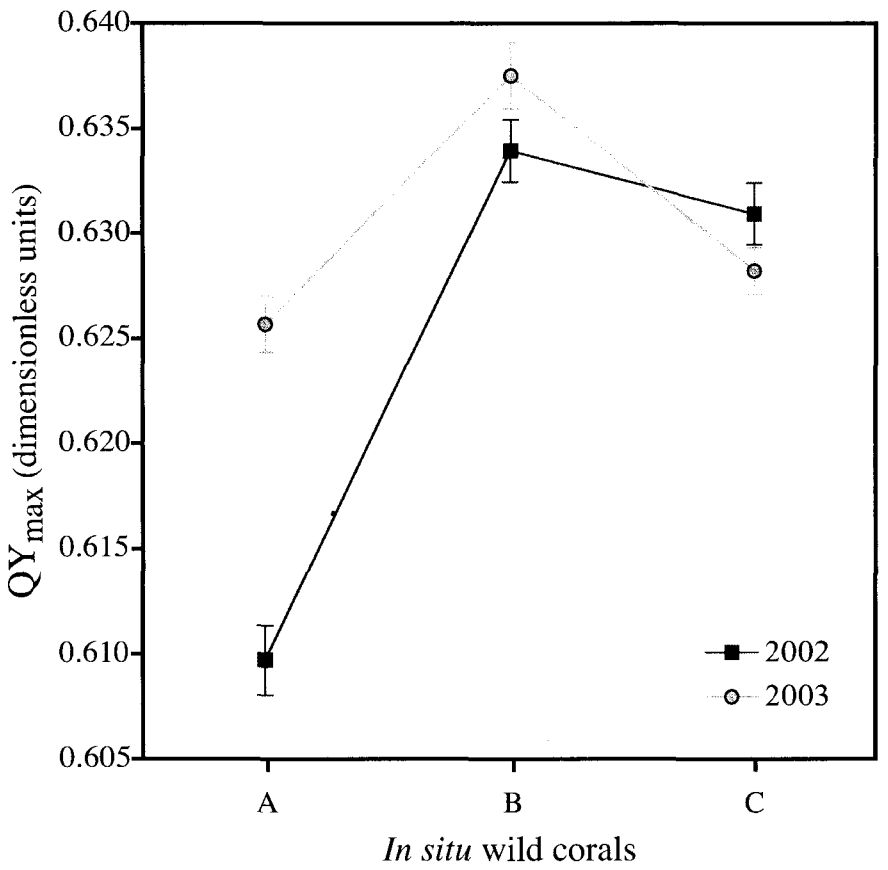


**Figure 4.** Quantum yield (QY) (dimensionless), in light-adapted colonies (n = 3) on nearby Conch Reef in 2002 and 2003 from random locations on three colonies. *Error bars* are the standard error of the mean, which are the average at random locations on the colonies. Data were arcsine transformed prior to computations and then back-transformed for graphical portrayal.



*PAR and Quantum Yield (2002 & 2003)*

A regression analysis resolved QY to be dependent on PAR (Figure 5 A & B).

*Chamber Temperature, Quantum Yield and PAR (2002)*

The analyses revealed the covariate chamber temperature to be a significant effect ($p = 0.013$). It was found, however, that for this data set the covariates PAR and chamber temperature are correlated (Spearman r = - 0.24, $p = 0.001$).

*Coral Bleaching Flow Chamber Velocity Profile Analysis (2002)*

Within all coral samples, speeds measured within the downstream sector (d) appeared less than 7.3 cm $s^{-1}$. Due to the highly turbulent nature of the downstream sector (d), the speeds reported in Table 3 are a snapshot, and do not show an increase of speed with height over the colony. The average flow velocities within a range of sectors and heights were collectively analyzed for all coral colonies (n = 6) for the (8 day) treatment in 2002 (Figure 6). Three factors were analyzed across the corals surface: colony, sector and height. A three-way ANOVA (Kutner and others 2005) resolved significant differences within the flow velocities which were expressed as asymmetrical patterns across all treated corals, sectors and heights. Since the same colonies were used again in 2003, the same flow patterns should have been obtained, as the corals did not grow sufficiently (less than 2 mm) to affect the flow through the chambers. The factors colony, sector and height were all statistically significant ($p < 0.0005$) (Table 1), but the sector*height interaction term was not.

**Figure 5.** Relationship between PAR irradiance and quantum yield, (QY) (dimensionless), in light-adapted colonies in the flow chambers during 2002 (**A**) and 2003 (**B**). Note the statistically significant inverse relationship. Variance in QY from light fluctuations, while present, was not sufficient to mask the significant difference in QY between upstream and downstream sectors evidenced during the course of the heated chamber treatments.

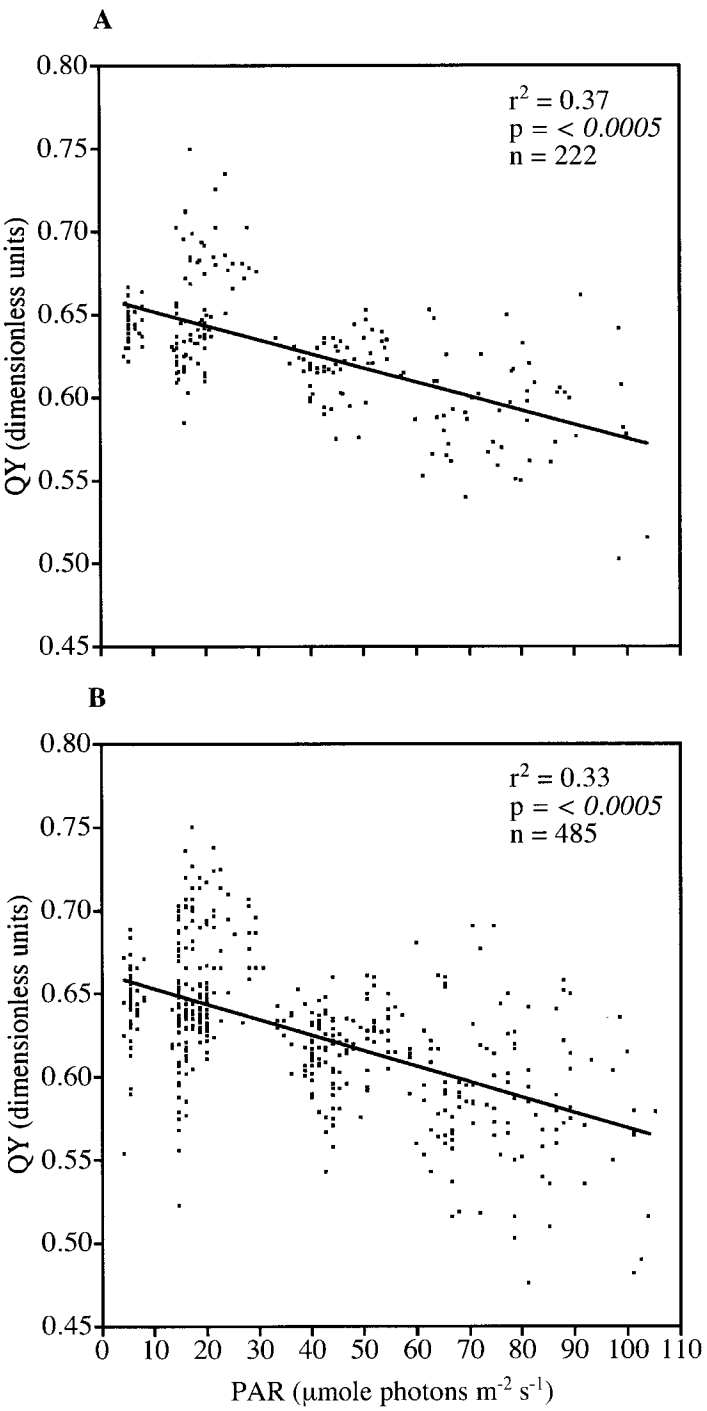
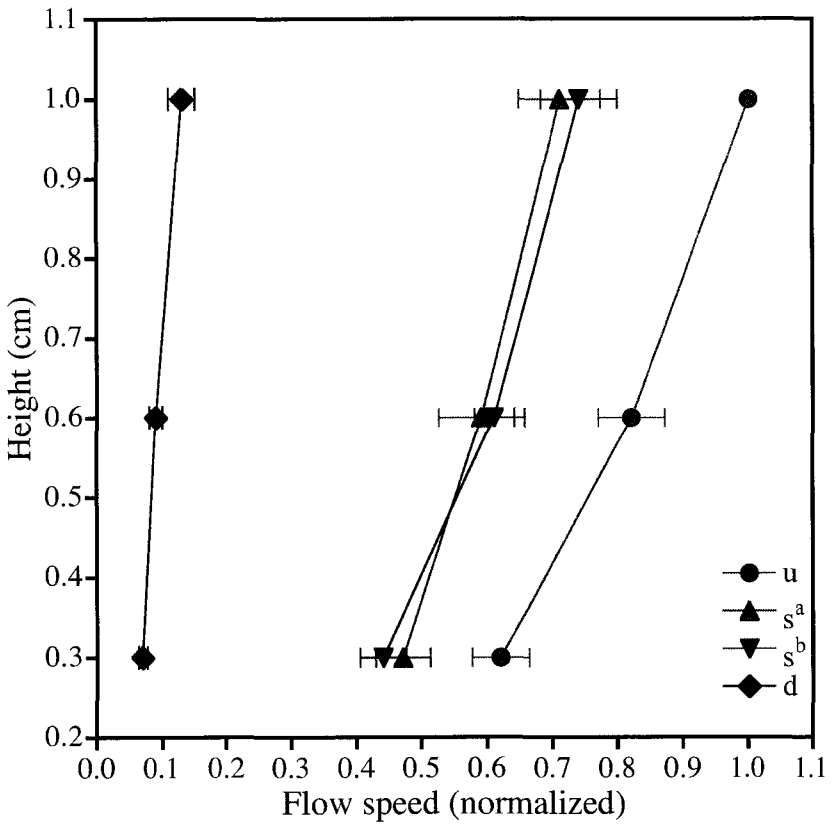


**Table 3.** The chamber flow-speeds (2002) were measured by tracking brine shrimp cysts using frame-by-frame video analysis in a collimated beam of light over coral colonies. Because of wake turbulence, flow speeds in the downstream sectors do not represent true speed at that height, as there was significant movement normal to colony surface. Rather the heights in all downstream sectors (d) represent the starting position of a tracked particle. Note that speeds are greatest on the upstream sector of the colony and lowest on the downstream sector.

| Sectors | Height above coral (cm) | Coral colony identifier | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| | | Flow speed (cm $s^{-1}$) | | | | | |
| Upstream (u) | 0.3 | 21.0 | 36.0 | 30.0 | 19.2 | 24.0 | 22.8 |
| | 0.6 | 31.8 | 42.0 | 42.0 | 25.2 | 30.0 | 30.0 |
| | 1.0 | 36.0 | 45.0 | 45.0 | 30.0 | 45.0 | 45.0 |
| Side[a] ($s^a$) | 0.3 | 21.0 | 24.0 | 24.0 | 15.0 | 16.2 | 15.0 |
| | 0.6 | 24.0 | 36.0 | 30.0 | 18.0 | 18.0 | 18.0 |
| | 1.0 | 30.0 | 39.0 | 33.0 | 24.0 | 22.8 | 24.0 |
| Side[b] ($s^b$) | 0.3 | 18.0 | 20.4 | 24.0 | 15.0 | 15.0 | 15.0 |
| | 0.6 | 21.0 | 30 | 33.0 | 18.0 | 24.0 | 25.2 |
| | 1.0 | 30.0 | 36 | 36.0 | 27.0 | 27.0 | 24.0 |
| Downstream (d) | 0.3 | 3.0 | 3.0 | 3.0 | 1.2 | 3.6 | 3.6 |
| | 0.6 | 3.6 | 3.0 | 3.6 | 2.4 | 6.0 | 4.8 |
| | 1.0 | 5.4 | 4.2 | 3.6 | 2.4 | 9.0 | 7.2 |

**Figure 6.** Chamber flow speeds relative to the upstream sector (1.0 cm) height in each coral colony for 2002. Heights were at the position mid-way up the side of the colony. *Error bars* are the standard error of the mean of normalized flow speed, where the flow speeds are normalized by the maximum flow recorded 1 cm above the coral at the upstream sector. Note that normalized flow speed is much greater on the upstream versus the downstream side of the colony. Data were arcsine transformed prior to computation and then back-transformed for graphical portrayal. All statistical analyses were performed using MINITAB (version 14).



*Quantum Yield and day (2002 & 2003)*

A regression analysis resolved QY to be dependent on Day (Figure 7 A & B).

*Upstream/Downstream Effects on Quantum Yield*

When the upstream sectors where compared to the downstream sectors, a consistent enhancement of QY on the downstream side was noted (Figure 8)

**Figure 7.** The relationship between day, upstream and downstream sectors and photosynthetic efficiency (QY) in colonies of *M. annularis* in flow chambers during 2002 (**A**) and 2003 (**B**). Note the statistically significant inverse relationship in both years. Two *in situ* field experiments using coral bleaching flow chambers with increased flows (*ca.* 40 cm $s^{-1}$) and elevated temperatures (*ca.* 2 °C above ambient) within coral colonies (n = 5) of *M. annularis*. Photosynthetic efficiency was higher in shallower velocity gradients on the downstream sectors of the coral. Data were arcsine transformed prior to computations. All statistical analyses were performed using MINITAB (version 14).

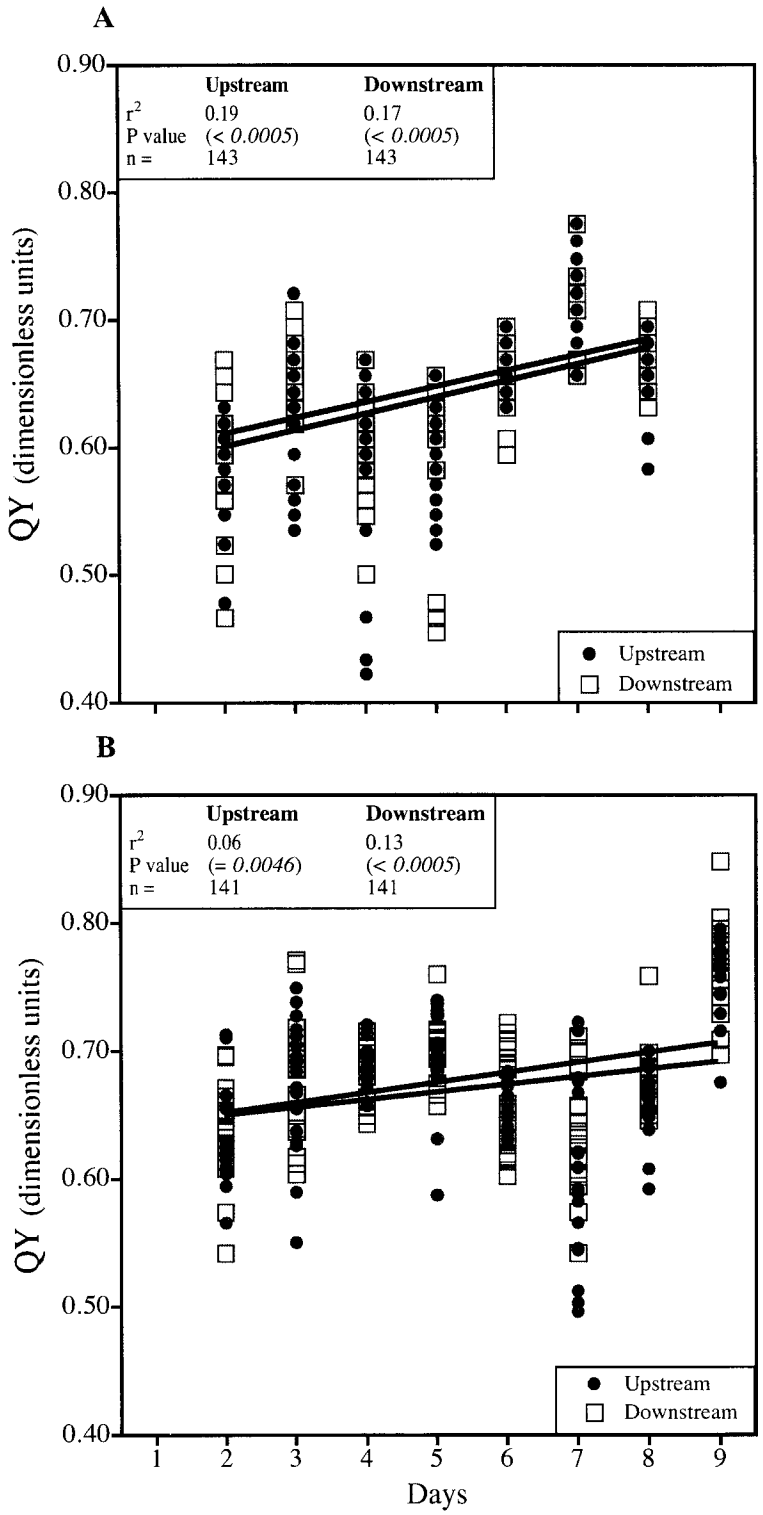
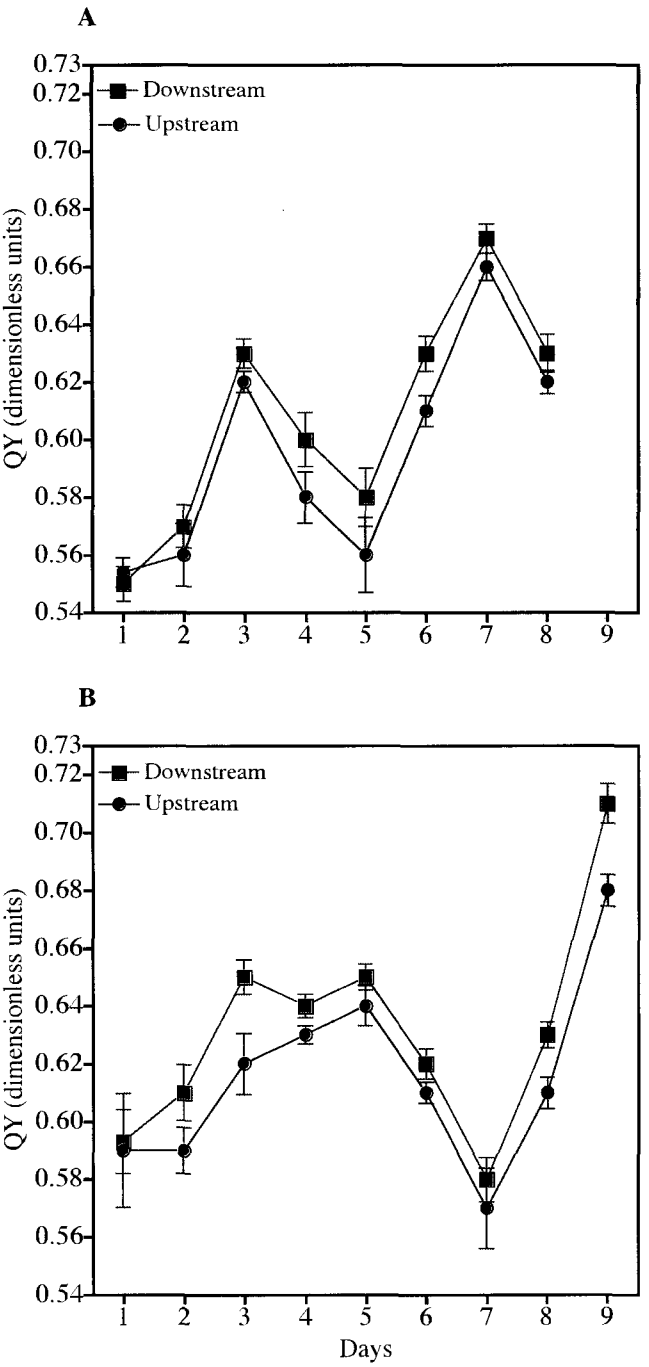


**Figure 8.** The daily up and downstream expression of the quantum yield, QY (dimensionless), during two *in situ* field experiments using coral bleaching flow chambers with increased flows (*ca.* 40 cm $s^{-1}$) and elevated temperatures (*ca.* 2 °C above ambient), within light adapted coral colonies (n = 5) of *M. annularis*, (**A**) 2002 and (**B**) 2003. Data were pooled for up and downstream sectors (n = 2) per day (n = 8 in 2002 & n = 9 in 2003). *Error bars* are standard error of the mean as reported in the analysis of covariance. Means represent the average of the sectors per day in all treated corals. Data were arcsine transformed prior to statistical computations and back-transformed for graphical portrayal. All statistical analyses were performed using MINITAB (version 14).



# DISCUSSION

The water-flow regime of Florida Keys reef slopes includes high frequency inputs from internal waves that contain dissolved nutrients, plankton, increased dissolved oxygen, and enhanced flow rates (Leichter and Miller 1999; Leichter and others 2003). A growing body of evidence supports the hypothesis that enhanced water-flow prevents the accumulation of damaging oxygen byproducts (derived form photosynthesis) in the soft tissues of coral colonies (Finelli and others 2006; Lesser 1996; Lesser and others 1994; Nakamura and van Woesik 2001; Nakamura and others 2003; Patterson 1992).

This study demonstrates a flow-modulated asymmetric distribution of photosynthetic efficiency over a coral colony (Figure 8) when corals are exposed to increased temperatures. These results are a logical extension of recent experimental findings that photosynthesis is modulated by oxygen efflux from coral tissue that in turn is modulated by flow (Done and others 2003; Downs and others 2002; Finelli and others 2006; Lesser 1997). Although thermal stress is viewed as the primary cause of coral bleaching, environmental factors including those of anthropogenic origin can act synergistically by lowering the *threshold temperature* at which coral bleaching can occur (Lesser 2006). The abiotic factor solar radiation has the greatest influence on thermally induced coral bleaching, both visible and ultraviolet components (UVB, UVA and in particular UVR) (Lesser 2006; Shick and others 1996).

Several studies have focused on water-flow, coral reef bleaching and enhanced rates of resistance and recovery to bleaching events (Done and others 2003; Finelli and others 2006; Nakamura and van Woesik 2001; Nakamura and others 2003; West and Salm 2003). For example, Nakamura and others (2003) experimentally bleached samples of *Stylophora pistillata* (Esper, 1797) and noted that recovery rates changed in accordance with the rate of water-flow over the coral. During a seven week manipulation, the number of zooxanthellae (per $cm^2$) and Chl *a* concentration ($\mu g\ cm^{-2}$) in moderate flow conditions of *ca.* 20 cm $s^{-1}$ demonstrated a rapid recovery following an initial three weeks of stasis post-bleaching. Colonies subjected to low-flow treatments ( 3 cm $s^{-1}$) showed only a slight change in their condition. The authors stated that moderate to high water-flow rates may facilitate a partial recovery from a bleaching event. They also suggested the mechanisms that lead to the dysfunction of the algal-coral symbiosis in the first instance may be driven by the limiting processes of mass transfer (Nakamura and van Woesik 2001). Nonetheless, whether this observed flow effect occurs indirectly through reduced temperatures or directly through modulation of gas exchange still needs to be addressed (Finelli and others 2006; Nakamura and van Woesik 2001).

***A Cautionary note on the use of Chlorophyll Fluorescence data***

The low cost and non-invasive nature of *in situ* fluorometry have helped establish it as the preferred technique for probing photosynthetic performance (Baker and Oxborough 2004). There are a number of potential heterogeneities in the response of chlorophyll fluorescence, and the assessment of *absolute values* of these fluorescence parameters is complicated by various factors. For a review of this subject, see the

following sources: (Baker and Oxborough 2004; Schreiber 2004). Nonetheless, PAM fluorometry is a valuable tool in assessing both photosynthetic performance and the extent to which it is limited by photochemical and non-photochemical processes. For this study, we used these data to examine *relative changes* as opposed to *absolute values*; that is to detect and follow changes in photosynthetic performance within a changing environment across the entire coral colony within the chamber over time, and to compare a range of morphologically dissimilar samples under the same environmental conditions.

There now exists new insight into the relationship between flow and photosynthesis (Finelli pers. comm. 2006). The intracolony variations of oxygen concentration, photosynthesis and flow speed were measured within a number of coral colonies (≤ 15 cm maximum dimension), under *in situ* ambient conditions. Transplanted corals were sampled from the same reef system as this study and placed perpendicular to the dominant tidal flow. The up/downstream facets of three coral species both transplanted and undisturbed, were measured for intracolony variation in photosynthetic efficiency, oxygen concentration, and flow speeds. Finelli (pers. comm. 2006) used a diving PAM fluorometer and rapid light curve (RLC) techniques to estimate the relative electron transport rate (rETR) through photosystem II at increasing levels of PAR. Finelli fitted the rETR vs. PAR relationship to a negative exponential curve that was parameterized by the initial slope alpha ($\alpha$) and the asymptotic maximum $rETR_{max}$ (Finelli pers. comm. 2006). *Siderastrea siderea* (Pallas, 1766) (mounding morphology) exhibited higher flow rates and $\alpha$ with lower $ETR_{max}$ located on the upstream compared to the slower flow rates downstream, and *Porites porites* (branching morphology)

demonstrated higher flow rates and $\alpha$ on the upstream side, with no effect on the $ETR_{max}$. In contrast higher $ETR_{max}$ was measured on the upstream side of *M. annularis* (flat plate morphology) similar to this study, but with no significant effect from flow (opposite to this study) or $\alpha$, but with a significant correlation between $\alpha$ and flow speed, Finelli (pers. comm. 2006) hypothesized that patchy distribution of bleaching within corals in a given system may be driven by the heterogeneity of flow and mass transfer, which is in agreement with the observations of Patterson and Price (1992).

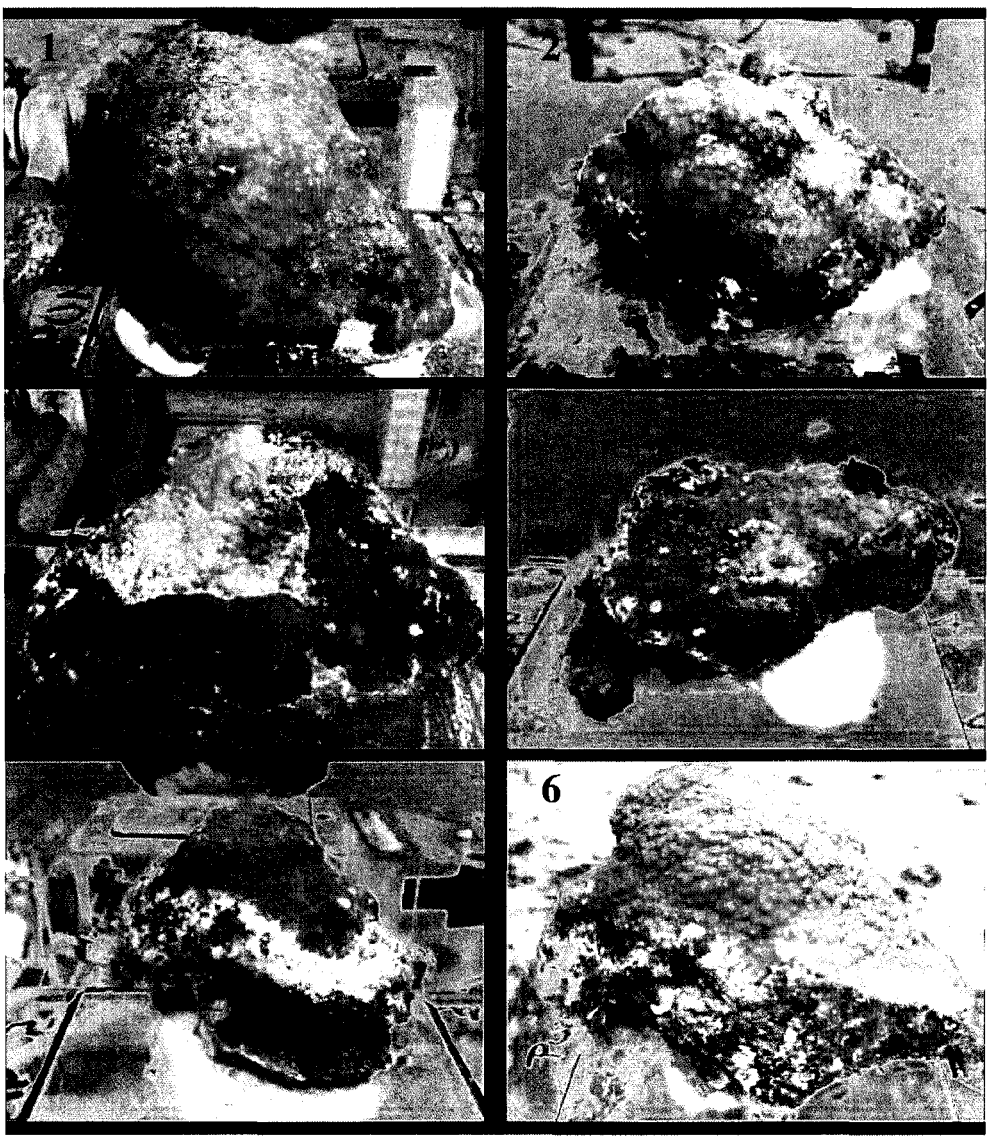
The effects of water-flow on corals have now been documented using portable flumes capable of delivering two flow regimes 0.1 cm $s^{-1}$ ($\pm$ 0.1 cm $s^{-1}$) and 15 cm $s^{-1}$ ($\pm$ 4 cm $s^{-1}$), in addition to *in situ* flow manipulations of undisturbed corals (Finelli and others 2006), and was carried out in the same reef system as this study. Several species were tested with the focus on *M. annularis* and *Agaricia agaricites* (Linnaeus, 1758). Chlorophyll fluorescence of light-adapted coral was measured within two flow conditions (flow and no-flow), oxygen concentrations and light levels. Both species had similar response patterns to light pre-adaptation with a QY at 300-400 $\mu$mole photons $m^{-2}$ $s^{-1}$, (the equivalent of unclouded summer noontime irradiance within this reef system); additional increases in irradiance in both flow and no-flow conditions substantially reduced QY (Finelli and others 2006). Flow manipulations revealed a significant flow response from *A. agaricites* at 300 $\mu$mole photons $m^{-2}$ $s^{-1}$, whereas *M. annularis* demonstrated no such response, with QY remaining the same in both flow and no-flow treatments at each light level. Interestingly Patterson and others (1991) found a high level of flow sensitivity within *M. annularis* during the processes of respiration and photosynthesis.

Within the Finelli and others (2006) study, the oxygen and flow manipulations resulted in *ca.* 20% decrease in QY (dimensionless) from *ca.* 0.26 in normoxic/flow to *ca.* 0.21 in the hyperoxic/flow treatments. Finelli and others (2006) found that when hyperoxic and increased flow conditions occurred simultaneously, the diffusive boundary layer (DBL) is reduced and the oxygen concentration gradient could be suppressed or even reversed. This combination resulted in a depressed QY in flow relative to the no-flow treatments, and could potentially result in an elevated oxygen concentration within the coral tissue. Conversely, QY was elevated during normal oxygen concentrations and in flow relative to no-flow treatments (Finelli and others 2006). It is worth noting the flume design of Finelli and others (2006) would only allow a *ca.* 3 $cm^2$ patch of coral tissue to be examined per treatment and therefore could not account for up/downstream measurements across the coral head as a whole.

This investigation documents flow-modulated asymmetric patterns of photosynthetic efficiency within mounding and flat plate morphologies of *M. annularis*. Using *in situ* unidirectional flow chambers, controlled temperatures and systematically measuring chlorophyll fluorometry, the same corals colonies (n = 5) were treated over two field seasons. These results demonstrate that the physical location of elevated and/or reduced QY within individual treatments is modulated by water-flow, with the highest QY values found on the downstream coral sectors. Given the various morphotypes and dimensions of the individual coral treatments (Figure 9), we would expect the water-flow dynamics within each chamber to be unique to that particular set-up.

**Figure 9.** Coral colonies of *Montastrea annularis* (1) thru (6). Given the different morphotypes and overall dimensions of the individual coral colonies, water-flow dynamics within each chamber should be unique to that particular coral/chamber combination. It is therefore of interest that all the corals (at least at the cellular level) appear to be responding in a similar fashion.

Colonies; 1, 3 and 6 - dome-like, 2 - *raised* flat-plate, 4 and 5 - flat-plate.



It is therefore of interest that all the corals (at least at the cellular level) appear to be responding in a similar fashion.

These results revealed the negative effects of both PAR and day on photosynthetic efficiency (2002) and a negative effect of PAR and a positive effect of day on the same (2003). We did not, however, discern a significant difference in these positive/negative interactions between up and downstream sectors within both years. The biological significance of increased (and decreased) photosynthetic efficiencies across the organism as a whole could have many impacts. In areas of reduced flow oxygen levels may potentially be increasing in concert with reduced mass transport through thicker boundary layers. The effect of a flow-limit upon effective prey capture (Patterson 1984; Patterson 1991; Sebens and others 1998), causing a shift in the location of prey capture from upstream to downstream, may deprive an already stressed group of polyps within a colony of needed energy.

This spatial asymmetry raises two points of interest, the greater exploitation of the incident radiation by the photosynthetic apparatus of downstream polyps experiencing less turbulence and slower water-flow speeds when compared to upstream sectors, and the concept of a water-flow threshold, above which photosynthetic efficiency may be compromised. These observations are of particular interest as photosynthesis (and therefore passive metabolic diffusion processes) were taking place at higher rates through thicker boundary layers at shallower velocity gradients on the downstream sectors, when compared to steeper velocity gradients and thinner boundary layers on upstream sectors.

In addition to the manipulated treatments, the photosynthetic efficiency (light-adapted quantum yield) was measured of undisturbed corals (n = 3) during 2002 and 2003. The same undisturbed corals were used in both years and were of the same size range and morphotype as the treatments. Within all of the *in situ* corals the factor coral was significant but sector was not, suggesting the overall flow regime experienced by these wild corals was multidirectional and of a similar magnitude (Figure 4). Comparing the photosynthetic efficiency of the experimental treatments to the *in situ* corals (by year) revealed different ranges of QY, particularly during 2002. Interestingly, within this study the chamber-generated velocity gradients (Figure 6 and Table 1) were comparable to the previously reported ambient flow speeds for the same reef system (Finelli and others 2006). Instantaneous flow speed measurements 5 cm above the coral head in ambient flows were routinely < 20 cm $s^{-1}$ and ranged up to a maximum of 30 cm $s^{-1}$. Mean flow speeds over 3 minute periods ranged from *ca.* 1.8 to 5.2 cm $s^{-1}$ (Finelli and others 2006), with longer observations revealing flow speeds of > 80 cm $s^{-1}$ at 5 cm above the corals (Finelli pers. comm. 2006). Therefore given the similar flow speeds within this study between the two environments, that is the chamber and those experienced by the *in situ* corals, the difference in photosynthetic efficiency may instead be attributable to either a flow/temperature-induced stress response or to the unidirectional nature of the chamber flow regime.

*Chamber Flow Velocity Reynolds numbers*

All six coral colonies possessed Re numbers between 13,440 – 45,000 (Table 4), which suggests a relatively high rate of metabolic processes were possible over the entire surface of the coral and not just the downstream sectors as described in the results. If the same disparity within the expression of QY between high and low areas across small coral colonies scale up to entire reefs, then the output of photosynthesis is cycling between high and low as water flow oscillates over the reef driven for example, by storm and waves orbitals or the effect that the reef structure may have on concentrating, and by default accelerating water flow velocities, along vertical profiles of reef channels.

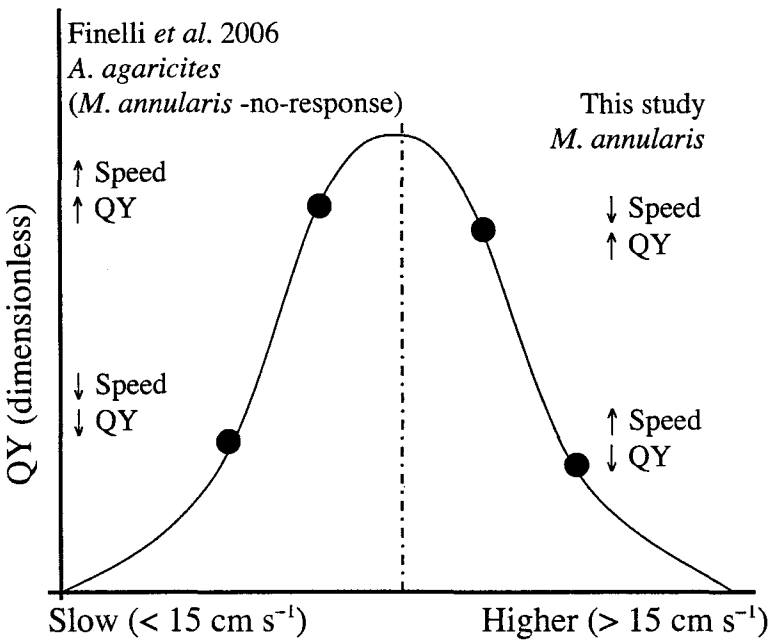
**Table 4.** To further characterize the nature of the flow régime over the colonies, we used the Reynolds number (Re), calculated from flow speed ($u$), colony head height ($W$), and momentum diffusivity (kinematic viscosity) of the fluid ($v$): Re = $uW/v$.

| | Coral colony identifier | | | | | |
|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** |
| Re | 29,600 | 16,400 | 23,400 | 17,360 | 33,000 | 29,340 |

Elevated rates of photosynthesis within steeper velocity gradients were found on upstream faces of *P. porites* and *S. siderea* when compared to downstream faces and shallower velocity gradients. Ambient flow speeds for *M. annularis* were measured at 3.8 cm $s^{-1}$ (± 1.6 cm $s^{-1}$) over upstream faces and 3.1 cm $s^{-1}$ (± 3.7 cm $s^{-1}$), over downstream faces (Finelli pers. comm. 2006). The findings of higher rates of photosynthesis within the upstream side (higher flow velocities) of a coral colony are diametrically opposite to the findings of this study as are their findings of no significant difference in flow speed or photosynthetic rate for *M. annularis* (Finelli pers. comm. 2006).

Why did we not observe higher QY in the faster flow over the upstream sectors of the manipulated corals within this study? The flow speeds reported here ranged from *ca.* 1.2 – 45.0 cm $s^{-1}$ at three heights of *ca.* 0.3, 0.6 and 1.0 cm, across four separate sectors. The significant difference between the flow velocities within this study and Finelli's could be explained by the existence of a yet to be identified flow threshold ($FT_{max}$) (Figure 10.). If an upper threshold or flow-limit exists above which a coral's metabolic function becomes impaired, and these manipulated flow speeds.

**Figure 10.** A conceptual model of the proposed affects of a Flow Threshold ($FT_{max}$), on temperature-stressed corals contrasting quantum yield (QY) expression (this study), and Finelli and others (2006).



breached this notional flow-limit, this situation may explain finding a higher QY located in the slower flow on the downstream sectors (this study), compared to Finelli (pers. comm. 2006) higher QY located in the faster flow on the upstream faces.

An increased water-flow over a particular coral sector would produce a thinner boundary layer and improved passive diffusive rates (Patterson 1991; Patterson and Sebens 1989). The effect of a sustained increase in metabolic rate and the existence of a $FT_{max}$ that could impede metabolic processes for example, restricting the photosynthetic machinery at a cellular level and/or affecting the regulation of *hsp*70 synthesis warrant further investigation. It has been established that increased water-flow can inhibit the build-up of oxygen and/or oxygen radicals (Downs and others 2002; Jones and others 1998; Lesser 1997; Nakamura and van Woesik 2001; Nakamura and others 2003). We now have data that suggests a detrimental impact from increased flow on the up-regulation of stress protein synthesis (*cf.* Chapter 3). Following sustained periods of enhanced flow, irradiance and thermal stress, a yet-to-be determined physiological threshold for example, flow may have a limiting effect on the organism during the accompanying periods of photosynthesis and increased energy expenditure required for molecular chaperone synthesis. This subject will be addressed further in Chapter 3.

Quantum yield within all treatments appeared to have a dependence upon chamber temperature and PAR irradiance. The analyses revealed higher values of QY during periods of increased chamber temperature and/or depressed irradiance. As anticipated, a strong dependence of QY when regressed against PAR (Figure 5 A & B)

was similar to that found in other studies *c.f.* (Finelli and others 2006; Warner and others 2002). In 2002, the lowest values of QY were recorded on day two and five (depressed chamber temperature), in 2003 on day two (highest irradiance), and on day seven (depressed ambient temperature and highest irradiance). During the 2002 treatment, the daily temperature range within the chamber was *ca.* 26.5° C - 28.5° C. Minco foil heaters maintained a constant temperature offset of ambient + 2° C, but the Plexiglas chambers were not insulated; therefore the water temperatures within the chambers were coupled to temperature changes that occurred during both deployments, notably from internal waves. Initial analyses identified that for every 1.0° C rise in the water temperature of the chamber, QY increased by 0.03 ± 0.01, however, further investigation revealed a correlation between chamber temperature and PAR irradiance within this data set. Since photosynthetic yield is strongly dependent upon irradiance, as found by this study and others for example, (Finelli and others 2006; Warner and others 2002), we propose this apparent dependence of QY on the temperature within the chamber may simply be due to the correlation of the covariates PAR, irradiance and chamber temperature. Nevertheless, we suggest this limited but transitory range of chamber temperatures may have modulated the metabolism of the treated corals as opposed to presenting a stable and potentially damaging stressor. A longer-term study using heated flow chambers may elucidate the effects of a limited but transient temperature range in a flow regime similar to the one used in this study.

Internal tidal bores are prevalent throughout the Florida keys reef tract (Leichter and others 2003; Leichter and others 1996) and may explain the rapid ambient

temperature anomalies observed at Conch Reef throughout both years of these field studies. The arrival of internal bores on the reef slope is associated with semidiurnal internal tides, sharp temperature decreases of > 5.4° C, and increased salinities of > 0.6 ‰ over short time periods of 1-20 minutes. The effects of these internal waves within the Florida reef system have been well documented (Leichter and Miller 1999; Leichter and others 1998; Leichter and others 1996; Smith 1983). Measurements in the range of *ca.* 10 - 40 fold increases in nutrient concentrations and increases of one to two orders of magnitude in nutrient flux relative to ambient non-bore conditions, have been documented within the same reef-system as this study (Leichter and others 2003; Leichter and others 1996). These short-term changes were accompanied by the rapid commencement of upslope flows of *ca.* 10-30 cm $s^{-1}$, 1-15 m above the bottom (Leichter and others 1996). These dynamic events appear regularly at Conch Reef from May through November, with the highest activity measured in July through September (Leichter and others 2003; Leichter and others 1996). The range of these temperature anomalies and specifically the rapid onset of increased flow rates correspond to the personal observations of all divers in both field seasons.

There are several advantages and disadvantages in reusing coral samples for both field seasons. For the second field season, the coral samples' response may not be analogous to that measured in the initial field season. There is the possibility these corals had not fully recovered from the 2002 treatment, although the divers reported that all corals appeared to have a healthy pigmentation, mucus production and were feeding prior to their removal for the second field season. Compared to the initial harvesting

techniques, the re-removal process appears less stressful and carries the advantage of the direct comparison of the same samples across both field seasons. The re-use of these samples is desirable for data comparison, robustness and experimental design. Deploying the same specimens also carries the advantage of acclimation to ambient seawater temperature, irradiance and nutrients prior to the installation of the corals into the chambers. There is also the advantage of using less reef-building coral to conduct *in situ* experiments, a specific concern of the Florida Keys National Marine Sanctuary when deciding on the issuance of coral collection permits. The results may underestimate the effects of the treatments since the corals were temporally removed form the flow of heated water to measure chlorophyll fluorescence as described above. Due to the expense of the diving PAM, it was not possible to allocate a separate instrument to each chamber in order to alleviate the need to remove corals from the chambers.

*Summary*

These experiments show that the QY of PS II is at a significantly reduced level on the upstream sectors (compared to downstream) of all treated coral colonies. *Montastrea annularis* may (at the molecular level) be passively suppressing the rates of photosynthesis within these spatially distinct high-flow areas or, increasing these rates in areas of lower water-flow, nonetheless, these processes appear to be sustained, occurring over a medium time-scale within the same colony and at two different times of the year. Water-flow might now be likened to an *agent provocateur* and posited to join the ranks of other bleaching conspirators (for example, solar radiation and oxygen toxicity) that act synergistically with temperature, in reducing the threshold at which thermal anomalies stimulate coral stress. We anticipate the stress protein analysis from tissue removed throughout this study will reveal a similar spatial asymmetry and allow for further inferences to be made with regards to the effect of flow velocities on stress protein synthesis and $FT_{max}$. With respect to coral systems, water-flow related stress is now being proposed as a viable co-stressor at the polyp level worthy of further investigation.

# LITERATUTE CITED

Baker NR, Oxborough K. 2004. Chlorophyll fluorescence as a probe of photosynthetic productivity. In: Papageorgiou GC, editor. Chlorophyll a fluorescence: A signature of photosynthesis. Netherlands: Springer. p 65-82.

Banaszak AT, Trench RK. 1995. Effects of ultraviolet (UV) radiation on marine microalgal invertebrate symbioses. I. Response of the algal symbionts in culture and in hospite. Journal of Experimental Marine Biology and Ecology 194:213-232.

Ben-Haim Y, Banin E, Kushmaro A, Loya Y, Rosenberg E. 1999. Inhibition of photosynthesis and bleaching of zooxanthellae by the coral pathogen *Vibrio shiloi*. Environmental Microbiology 1:223-229.

Ben-Haim Y, Thompson FL, Thompson CC, Cnockaert MC, Hoste B, Swings J, Rosenberg E. 2003. *Vibrio coralliilyticus* sp. nov., a temperature-dependent pathogen of the coral *Pocillopora damicornis*. International Journal of Systematics Evolution and Microbiology 53:309-315.

Brown BE. 1997. Coral bleaching: Causes and consequences. Coral Reefs 16(Suppl.):S129-S138.

Brown BE, Downs CA, Dunne RP, Gibb SW. 2002a. Exploring the basis of thermotolerance in the reef coral *Goniastrea aspera*. Marine Ecology Progress Series 242:119-129.

Brown BE, Dunne RP, Goodson MS, Douglas AE. 2002b. Experience shapes the susceptibility of a reef coral to bleaching. Coral Reefs 21:119-126.

Buddemeier RW, Fautin DG. 1993. Coral bleaching as an adaptive mechanism: a testable hypothesis. BioScience 43:320-326.

Cole J. 2003. Dishing the dirt on coral reefs. Nature 421:705-706.

Done TJ, Turak E, Wakefirld M, De'ath G, Kininmonth S, Wooldridge S, Berkelmans R, Van Oppen M, Mahoney M. 2003. Tea sting bleaching resistance hypotheses of the 2002 Great Barrier Reef bleaching event: a contribution to the Transforming Coral Reef Conservation initiative and toolkit. Australian Institute of Marine Science. Final report to The Nature Conservation.

Downs CA, Fauth JE, Halas JC, Dustan P, Bemiss J, Woodley CM. 2002. Oxidative stress and seasonal coral bleaching. Journal of Free Radical Biology and Medicine 33(4):533-543.

Falkowski PG, Kobler ZS, Gorbunov MY, Kolber Z, editors. 2004. Development and application of variable chlorophyll fluorescence techniques in marine ecosystems. Netherlands: Springer. 757-778 p.

Finelli CM, Helmuth BST, Pentcheff DN, Wethey DV. 2006. Water flow influences oxygen transport and photosynthetic efficiency in corals. Coral Reefs 25:47-57.

Fitt WK, Warner ME. 1995. Bleaching patterns of four species of Caribbean reef corals. Biological Bulletin 189:298-307.

Gardella DJ, Edmunds PJ. 1999. The oxygen microenvironment adjacent to the tissue of the scleractinian *Dichocoenia stokesii* and its effects on symbiont metabolism. Marine Biology 135:289-295.

Gardella DJ, Edmunds PJ. 2001. The effect of flow and morphology on boundary layers in the scleractinians *Dichocoenia stokesii* (Milne-Edwards and Haime) and *Stephanocoenia michilini* (Milne-Edwards and Haime). Journal of Experimental Marine Biology and Ecology 256:279-289.

Gleason DF, Wellington GM. 1993. Ultraviolet radiation induces coral bleaching: evidence from field experiments. Nature 365:836-838.

Glynn PW. 1993. Coral reef bleaching: ecological perspectives. Coral Reefs 12:1-17.

Gorbunov MY, Falkowski PG, Kolber Z. 2000. Measurement of photosynthetic parameters in benthic organisms *in situ* using a SCUBA-based fast repetition rate fluorometer. Limnology and Oceanography 45(1):242-245.

Hoegh-Guldberg O. 1999. Climate change, coral bleaching and the future of the world's coral reefs. Marine and Freshwater Research 50:839-866.

Hughes T. 1994. Catastrophes, phase shifts, and large-scale degradation of a Caribbean coral reef. Science 265:1547-1551.

Jokiel PL, Coles SL. 1974. Effects of heated effluent on hermatypic corals at Kahe Point, Oahu. Pacific Science 28:1-18.

Jones RJ, Hoegh-Guldberg O, Larkum AWD, Schreiber U. 1998. Temperature-induced bleaching of corals begins with impairment of the $CO_2$ fixation mechanism in zooxanthellae. Plant, Cell and Environment 21:1219-1230.

Jones RJ, Kildea T, Hoegh-Guldberg O. 1999. PAM chlorophyll fluorometry: A new *in situ* technique for stress assessment in scleractinian corals, used to examine the effects of cyanide from cyanide fishing. Marine Pollution Bulletin 38(10):864-874.

Knowlton N, Mate JL, Guzman HM, Rowan R, Jara J. 1997. Direct evidence for reproductive isolation among the three species of the *Montastraea annularis* complex in Central America (Panama and Honduras). Marine Biology 127:705-711.

Knowlton N, Weil E, Weight LA, Guzman HM. 1992. Sibling species in *Montastraea annularis*, coral bleaching, and the coral climate record. Science 255:330-333.

Kutner MH, Nachtsheim CJ, Neter J, Li W. 2005. Applied Linear Statistical Models. Gorden B, editor. New York: McGraw-Hill/Irwin. 1396 p.

Leichter JJ, Miller SL. 1999. Predicting high-frequency upwelling: Spatial and temporal patterns of temperature anomalies on a Florida coral reef. Continental Shelf Research 19:911-928.

Leichter JJ, Shellenbarger G, Genovese SJ, Wing SR. 1998. Breaking internal waves on a Florida (USA) coral reef: A plankton pump at work? Marine Ecology Progress Series 166:83-97.

Leichter JJ, Stewart HL, Miller SL. 2003. Episodic nutrient transport to Florida corals reefs. Limnology and Oceanography 48(4):1394-1407.

Leichter JJ, Wing SR, Miller SL, Denny MW. 1996. Pulsed delivery of subthermocline water to Conch Reef (Florida Keys) by internal tidal bores. Limnology and Oceanography 41(7):1490-1501.

Lesser MP. 1996. Exposure of symbiotic dinoflagellates to elevated temperatures and ultraviolet radiation causes oxidative stress and inhibits photosynthesis. Limnology and Oceanography 41:271-283.

Lesser MP. 1997. Oxidative stress causes coral bleaching during exposure to elevated temperatures. Coral Reefs 16:187-192.

Lesser MP. 2006. Oxidative Stress in Marine Environments: Biochemistry and Physiological Ecology. Annual Review of Physiology 68(1):253-278.

Lesser MP, Weis VM, Patterson MR, Jokiel PL. 1994. Effects of morphology and water motion on carbon delivery and productivity in the reef coral, *Pocillopora damicornis* (Linnaeus): Diffusion barriers, inorganic carbon limitation, and biochemical plasticity. Journal of Experimental Marine Biology and Ecology 178:153-179.

Lopez JV, Kersanach R, Rehner SA, Knowlton N. 1999. Molecular determination of species boundaries in corals: Genetic analysis of the *Montastraea annularis* complex using amplified fragment length polymorphisms and a microsatellite marker. Biological Bulletin 196:80-93.

Nakamura T, van Woesik R. 2001. Water-flow rates and passive diffusion partially explain differential survival of corals during the 1998 bleaching event. Marine Ecology Progress Series 212:301-304.

Nakamura T, Yamasaki H, van Woesik R. 2003. Water flow facilitates recovery from bleaching in the coral *Stylophora pistillata.* Marine Ecology Progress Series 256:287-291.

Patterson MR. 1984. Patterns of whole colony prey capture in the octocoral, *Alcyonium siderium.* Biological Bulletin 167(3):613-629.

Patterson MR. 1991. The effects of flow on polyp-level capture in an octocoral, *Alcyonium siderium.* Biological Bulletin 180:93-102.

Patterson MR. 1992. A mass transfer explanation of metabolic scaling relations in some aquatics invertebrates and algae. Science 255:1421-1423.

Patterson MR, Price WS. Correlation of water motion with rates of intracolony coral bleaching; 1992; Santa Fe.

Patterson MR, Sebens KP, Olson RR. 1991. *In situ* measurements of flow effects on primary production and dark respiration in reef corals. Limnology and Oceanography 36(5):936-948.

Patterson MR, Sebens PK. 1989. Forced convection modulates gas exchange in cnidarians. Proceedings of the National Academy of Sciences 86:8833-8836.

Richardson LL, Goldberg WM, Kuta KG, Aronson RB, Smith GW, Ritchie KB, Halas JC, Feingold JS, Miller SL. 1998. Florida's mystery coral-killer identified. Nature 392:557-558.

Saxby T, Dennison WC, Hoegh-Guldberg O. 2003. Photosynthetic responses of the coral *Montipora digitata* to cold temperature stress. Marine Ecology Progress Series 248:85-97.

Schlichting HG, Klaus K, Egon M, Katherine O, Herbert J. 2000. Boundary Layer Theory: Springer Verlag.

Schreiber U. 2004. Pulse-Amplitude-Modulation (PAM) fluorometry and saturation pulse method: An overview. In: Papageorgiou GC, editor. Chlorophyll a fluorescence: A signature of photosynthesis. Netherlands: Springer.

Schrieber U, Bilger W, Neubauer C. 1994. Chlorophyll fluorescence as a nonintrusive indicator for rapid assessment of *in vivo* photosynthesis. In: Schulze ED, Caldwell MM, editors. Ecophysiology of Photosynthesis. Berlin: Springer. p 49-70.

Sebens KP, Grace S, Helmuth BST, Maney E, Miles J. 1998. Water flow and prey capture by three scleractinian corals, *Madracis mirabilis*, *Montastrea cavernosa* and *Porites porites* in a field enclosure. Marine Biology 131:347-360.

Sebens KP, Helmuth BST, Carrington E, Agius B. 2003. Effects of water flow on growth and energetics of the scleractinian coral *Agaricia teniufolia* in Belize. Coral Reefs 22:35-47.

Shashar N, Cohen Y, Loya Y. 1993. Extreme diel fluctuations of oxygen in diffusive boundary layers surrounding stony corals. Biological Bulletin 185:455-461.

Shashar N, Kinane S, Jokiel PL, Patterson M R. 1996. Hydromechanical boundary layers over a coral reef. Journal of Experimental Marine Biology and Ecology 199:17-28.

Shick JM, Lesser MP, Jokiel P. 1996. Effects of ultraviolet radiation on corals and other coral reef organisms. Glob. Change Biol. 2:527–45.

Smith NP. 1983. Temporal and Spatial Characteristics of Summer Upwelling along Florida's Atlantic Shelf. Journal of Physical Oceanography 13(9):1709-1715.

Thomas F, Atkinson M. 1997. Ammonium uptake by coral reefs: Effects of water velocity and surface roughness on mass transfer. Limnology and Oceanography 42:81-87.

Vogel S. 1996. Life In Moving Fluids: Princeton University Press. 467 p.

Warner ME, Chilcoat GC, McFarland FK, Fitt WK. 2002. Seasonal fluctuations in the photosynthetic capacity of photosystem II in symbiotic dinoflagellates in the Caribbean reef-building coral *Montastrea*. Marine Biology 141:31-38.

West JM, Salm RV. 2003. Resistance and resilience to coral bleaching: Implications for coral reef conservation and management. Conservation Biology 17(4):956-967.

## CHAPTER 2

# A NON-DESTRUCTIVE METHOD OF SAMPLING LIVE CORAL TISSUE, SINGLE POLYP-SAMPLE PREPARATION AND PROTEIN QUANTIFICATION FOR ASSESSMENT OF CORAL HEAT SHOCK PROTEINS

Submitted to Coral Reefs

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## ABSTRACT

Sessile marine invertebrates are routinely exposed to fluctuations in their environment such as temperature, irradiance, terrestrial run-off and water-flow. Many of these fluctuations can affect a coral reef at a range of scales from molecular to an entire reef system with resulting effects on growth and energetics, reproductive efficiency, protein damage and the loss of pigment or expulsion of the symbiotic dinoflagellate *Symbiodinium*. The induction and regulation of heat shock proteins (*hsps*), is a significant defense mechanism that can preserve metabolic function and foster recovery from short-term stress events. Adaptation from thermal histories can help corals in subsequent thermal insults. Current coral sampling methods often employ an invasive approach in the collection of coral tissue, cleaving off coral fragments (~ 100 $cm^2$) from live coral colonies that may already be in a stressed or poor condition. Subsequent protein preparative methods have to further purify the sample to account for contaminants, *e.g.*, $CaCO_3$ and coral mucus. In the present study, three methods were developed specifically to; (i) carryout single polyp time-series sampling on live coral colonies ($\leq$ 150 $cm^2$) (n = 6), without compromising the colonies or parallel studies on the photobiology of the colonies, (ii) develop a low volume (50-100 µl of coral tissue) protein recovery method for single coral polyps, and (iii) develop a low volume, high resolution protein quantification method. The preliminary testing of five separate protein preparation methods resulted in a range of total protein yields from 47 to 77 (ug) per

coral polyp. Subsequent SDS-PAGE and immunoblotting analysis on single coral polyps (n = 200), resolved as little as 87 pg of *hsp*70 per coral polyp. These results demonstrate that relatively large amounts of total protein ( $\bar{x}$ = 77 +/- 9 μg) are recoverable from single coral polyps. The new sampling method limits coral damage during collection, and may also reduce the susceptibility to disease and additional stress during and post sampling.

## INTRODUCTION

Stress protein synthesis is widely used as a biomarker to indicate past and real-time stress events. These molecular chaperones are also used in the study of an organism's potential adaptation to future stress, such as the delayed onset of stress protein synthesis due to an individual's thermal history (Buckley and Hofmann 2002; Hofmann 2005). Heat shock protein regulation and synthesis is well documented in the literature, and a number of cnidarians have been examined. Heat shock protein 70 (*hsp*70) has been resolved in the coral *Goniopora djiboutiensis* (Vaughan, 1907) (Sharp and others 1994) and the anemone, *Anemonia viridis* (Forsskål, 1775) which produced six different *hsps* isoforms when exposed to elevated temperatures or copper chloride (Miller and others 1992). A range of *hsps* were detected whilst resolving the highly abundant 33 kDa protein from the sea anemone *A. viridis*, as well as in the tropical corals *G. djiboutiensis, Goniopora pandoraensis* (Veron and Pichon, 1982) *and Goniopora stokesi* (Milne Edwards and Haime, 1851, (Bythell and others 1995), in addition to thermal experiments carried out on the sea anemone *Aiptasia pallida* (Verrill) and the Caribbean reef coral *Montastrea faveolata* Ellis and Solander, 1786 (Black and others 1995).

*Current Methods of Coral Polyp Sampling and Preparation for Physiological Studies*

Studies of coral physiology often require the collection of biological samples that rely heavily upon laboratory preparation and analysis to resolve the specific data of interest from coral samples, such as during bleaching and energetic studies. Sample collection can frequently involve cleaving off coral fragments (~ 100 $cm^2$), rendering the remaining colony potentially susceptible to disease and/or to additional loss of soft tissue bordering the fragmented zones. Typical examples of coral removal methods include hammer and chisel (Miller. K and Ayre 2004), steel hole punch (Toller and others 2001) and coring (Rowan and others 1997). When the coral polyp is the focus of a study, the very removal of the sample from the colony can causes contamination, *e.g.*, $CaCO_3$ or coral mucus. These contaminants then have to be accounted for by current sample preparative methods. At some point, the soft tissue has to be either homogenized with, or separated from the underlying $CaCO_3$ skeleton. Several methods to remove the soft tissue already exists, for example, freezing in liquid nitrogen whilst grinding (Bythell and others 1995; Downs and others 2002), jetting off the coral tissue with a water-pick (Ben-Haim and others 2003; Black and others 1995), and heating polyps in sample buffer (Downs and others 2002; Griffin 2005; Robbart and others 2004). Subsequent analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, however, frequently produces results that are difficult to interpret, primarily due to extensive lane smearing and non-specific binding. Corals contain high levels of lipids (Achituv and others 1994; Oku and others 2003; Yamashiro and others 1999) and polysaccharide (Ducklow and Mitchell 1979; Krupp 1985), both of which interfere with protein analysis by blocking the pores of SDS PAGE gels or altering the

molecular weight of proteins (Carpentier and others 2005; Saravanan and Rose 2004). Furthermore, the densitometry analyses of the resulting gels is often inappropriately performed (Rossi and Snyder 2001), as the limitations of the detection system are not routinely taken into account in that the scanned image-data requires further re-calibration. Therefore, more often than not, protein concentration is underestimated.

***Need for Low Volume, Minimally Invasive Sampling of Coral Tissue***

Coral reefs are in a state of global decline (Hughes and others 2003; Jackson and others 2001; Pandolfi and others 2003). Approximately 58 - 70% of coral reefs are reported to be under significant anthropogenic pressures with up to 60% potentially lost by 2030 (Gardner and others 2003; Hoegh-Guldberg 1999; Hughes and others 2003; Wilkinson and others 1999). Coral reef systems that endure chronic environmental and anthropogenic disturbance can exhibit a range of deleterious impacts, for example depressed reproductive effort and growth rates, elevated frequency and severity of diseases, and mass mortalities (Hoegh-Guldberg 1999; Knowlton 2001; Nystrom and others 2000). Coral biologists that study physiological processes of corals are under increasing pressure to minimize destructive use of live coral in lab and field experiments. There is also a need to sample small volumes of tissue from live corals to obtain physiological snapshots of reef health (Yamashiro and others 1999).

In this present study, a non-destructive single coral polyp sampling method was developed. Five sample preparation/recovery methods were tested on a range of pre- and post-stressed coral polyps to establish the highest possible protein recovery (per polyp),

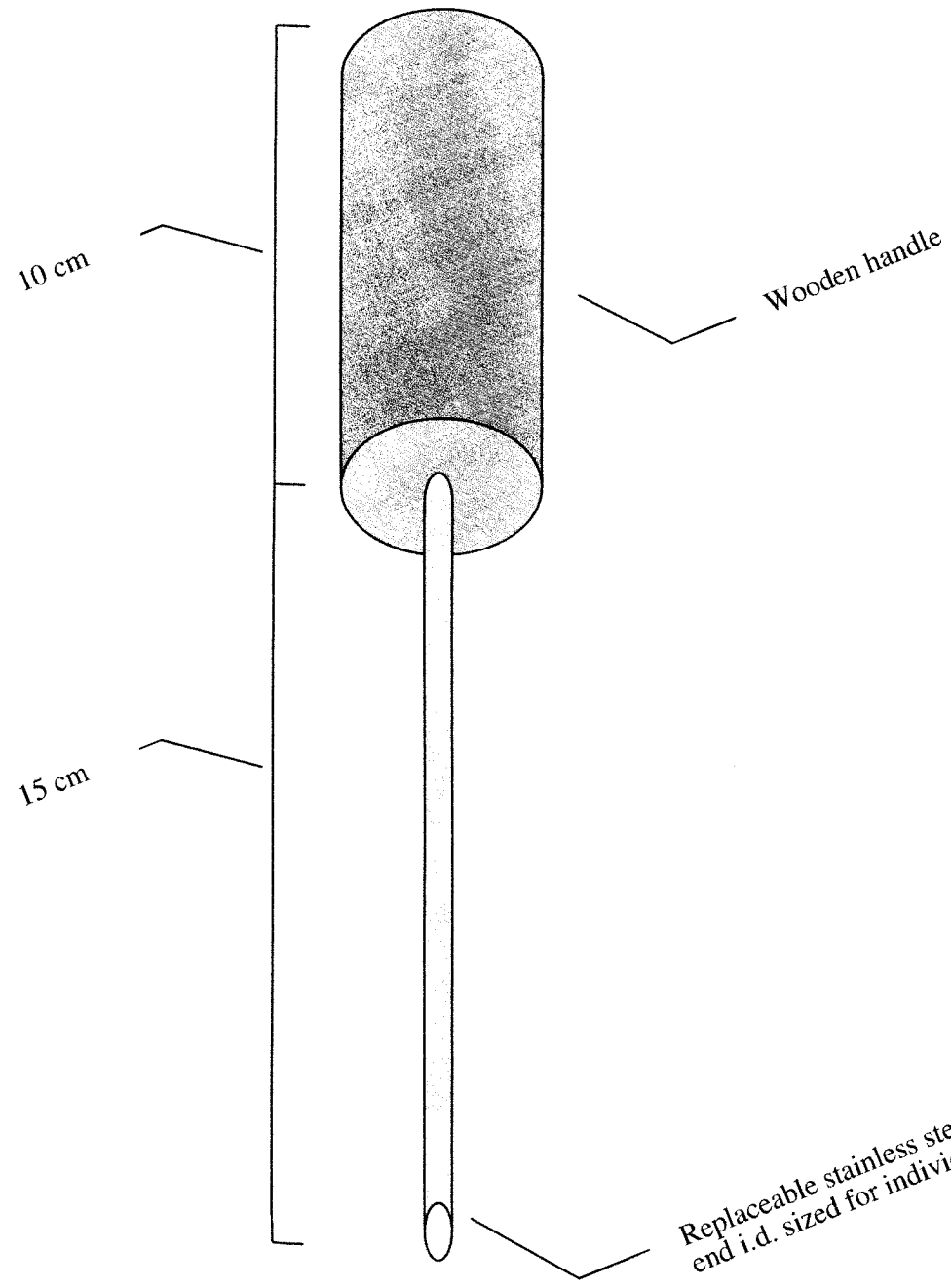
and a simplified protein quantification method was established, all of which where specifically designed for the low sample volumes associated with solitary polyps.

# MATERIALS AND METHOD

## *Single Coral Polyp Sampling Method*

Dependent on the species of interest, the diameter of the polyps will define the size of each sample. Coral samples were excised underwater whilst SCUBA diving, from coral colonies (n = 6) of *Montastrea annularis* (Ellis and Solander, 1786) with a hollow-point stainless steel polyp-spike (i.d. 4 mm) similar to a fish-tagging tool (*cf.* Figure 1). If the tip became damaged or dull during use, it was replaced. A constant pressure (with rotation) was applied until the tip of the polyp-spike bottomed-out on the underlying $CaCO_3$ skeleton. The spike was then slowly withdrawn and the polyp transferred into a pre-labeled (5 ml) syringe. Once at the surface after the collection dive, samples were transferred into labeled Eppendorf tubes containing a cocktail of proteases inhibitors (P8340, Sigma) to inhibit protein degradation (Cox and Lewis 2003; Griffin and Bhagooli 2004), diluted by volume to a ratio of 1:100 (inhibitor:sample). Samples were then stored in the dark with ice during the transit back to shore and subsequently transferred to liquid nitrogen for -80° C storage.

**Figure 1.** A diagrammatic illustration of a stainless steel polyp-spike, similar to a fish-tagging tool. To excise coral polyps, a constant pressure (with rotation) was applied, until the tip of the polyp spike bottomed out on the underlying $CaCO_3$ skeleton. The spike was then slowly withdrawn and the polyp was transferred into a pre-labeled (5 ml) syringe (not shown). The outside diameter of the polyp spike was 5 mm and was determined by the size of the polyps for *M. annularis*.



***Protein Recovery from Single Coral Polyp Preparation (Methods)***

Five different methods were assessed. Each method was assessed in relation to time and protein yield. The first method, samples were processed according to the procedure of Downs and others (2000), with minor modifications for low sample volumes. Samples (n = 5) were thawed and the tissues disrupted by the use of sonication or repeated freeze-thaw cycles in liquid nitrogen with homogenization by an Eppendorf grinder (Kontes No. 749521-1500). Freshly prepared sample buffer, twice the volume of the polyp was added, and the sample heated to 95° C for 6 - 10 minutes with repeated mixing. Samples were then centrifuged at 13,000g, at 4° C for 15 minutes. The middle phase containing the coral proteins was removed, taking great care not to disturb the upper polysaccharide layer. Samples were then assayed in duplicate for total protein, with minor modifications to previously a published method (Ghosh and others 1988). Briefly, 1 μl volumes of sample, sample preparation buffer, and protein standard were spotted in duplicate onto Whatman (3M) filter paper, allowed to dry, and then stained for *ca.* 20 minutes in Coomassie blue (G250, Sigma). The filter paper was then de-stained in 50% methanol, 10% acetic acid and 40% ultra-pure water (v/v), until no visible background remained. The filter paper was dried, and the individual sample spots excised into discs of uniform size, using a paper hole punch. Duplicate blanks of filter paper were also prepared to compensate for any remaining background stain in the filter paper. Each stained spot was individually eluted from the paper into 1 ml of 1% sodium dodecyl sulfate (SDS) with agitation. Two hundred $\mu$l of each replicate was then transferred to a 96-well microtitre plate, and absorption read at 595 *nm*, using a microtitre plate spectrophotometer and the remaining samples were stored at –20° C until required.

For direct comparison of the next four methods, coral polyps (n = 20) were pooled together with a Laemmli reducing sample buffer (2 ml) (minus glycerol and bromophenol blue) (Laemmli 1970). Coral polyps were ground for one minute with a Teflon Eppendorf grinder, (Kontes No. 749521-1500), then boiled for 5 minutes and ground for a further 30 seconds. The polyp homogenate were spun for 10 minutes at 10,000g and the supernatant was removed and separated into four groups of five one polyp samples, equivalent to one polyp each. The following four methods of protein precipitation were tested:

ii) *Ammonia sulfate*: An equal volume of saturated ammonia sulfate was slowly added to the polyp homogenate, and the resultant mix rotated overnight at 4° C.

iii) *Acetone*: Four volumes of chilled (-20° C) acetone were added to the polyp homogenate, thoroughly mixed and incubated overnight at -20° C.

iv) *Trichloroacetic acid + Deoxycholate (TCA + DOC)*: An equal volume of chilled (20%) TCA containing (2%) DOC was added to the polyp homogenate and incubated overnight at -20° C.

v) *TCA + DOC + Acetone + 2-mercaptoethanol (2ME)*: Four volumes of acetone containing (15%) TCA + 1% DOC and 0.07% 2ME, were added to the polyp homogenate, thoroughly mixed and incubated for 3 hours at -20° C.

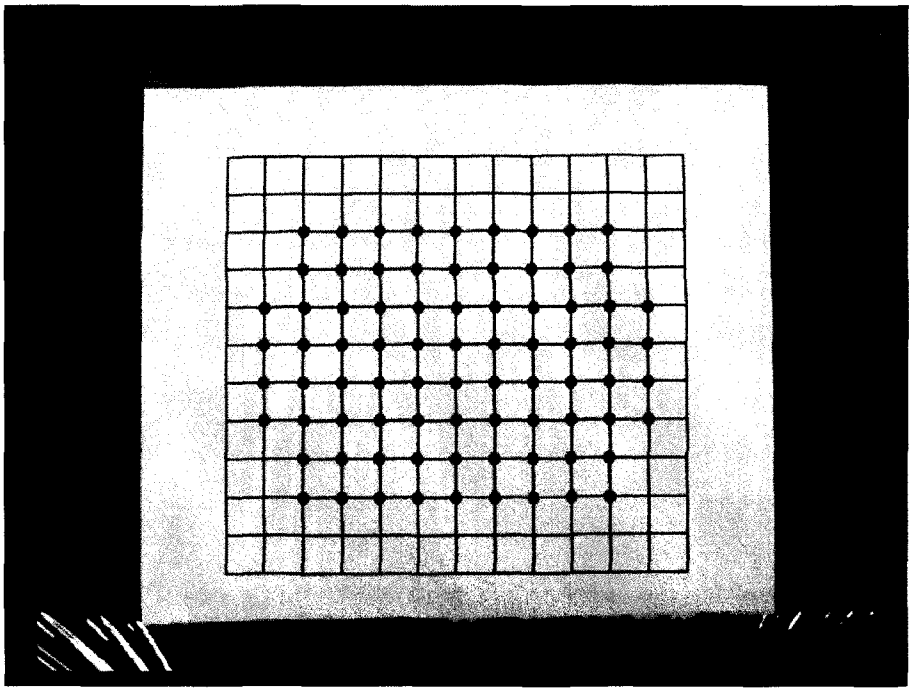
Following incubation, the homogenates were centrifuged at 15,000g for 20 minutes the supernatant was then removed from the pellet and discarded. The pellet was agitated in 500 µl of ice-cold (-20° C) acetone containing 0.07% 2ME, and centrifuged at

15,000g for 20 minutes. The supernatant was again removed and discarded and this process repeated two more times. The pellet was allowed to air dry for 10 minutes at room temperature then re-suspended in 25 µl of 8M urea and 75 µl of double strength Laemmli sample buffer, agitated and heated if necessary. Six µl was drawn off each (polyp) sample for protein quantification, and the remaining solution was stored (-20° C) until required.

***Total Protein Estimation***

A new method for total protein estimation was developed to assess total coral protein from a small sub-sample volume (2 µl), at low concentrations (< 200 µg/ml), and in the presence of a Laemmli sample buffer. This total protein estimation method had to be developed, so that a consistent amount of protein could be loaded onto SDS-PAGE gels for the quantification of *hsps*. Immobilon-FL (Millipore) Polyvinylidene fluoride membrane (PVDF), was activated in 100% methanol and subsequently placed in ultra-pure water for 5 minutes. The activated membrane was briefly dried on filter paper (Whatman) to remove surface water and then transferred directly onto dry, sterile Parafilm that was secured over a 1 $cm^2$ grid (*cf.* Figure 2). Before the membrane dried, 2 µl of each protein sample were added in duplicate to the membrane using the underlying grid as a guide.

**Figure 2.** The activated membrane (not shown) was dried briefly on filter paper (Whatman) to remove surface water and transferred directly onto a piece of dry, sterile Parafilm secured over a 1 $cm^2$ grid, to assist in placement of 2 µl aliquots.



For protein estimation, a logarithmic dilution series (3.12, 6.25, 12.5, 25, 50, 100, 200, 400, 800, and 1600 μg/ml) of acetone precipitated bovine serum albumin (BSA) and double strength reducing sample buffer (2 μl) was then added to the membrane. Following the application of samples and dilution series, the PVDF was air dried to allow the adhesion of the proteins, then reactivated in methanol and washed 3 times (5 minutes each) in ultra-pure water. The PVDF membrane was stained with freshly made Coomassie blue (G250, Sigma) for one minute and then de-stained in the dark with multiple changes of de-stainer (10% acetic acid, 50% methanol, 40% water), until the background was white. The de-stained membrane was allowed to dry, placed face down on the surface of the Odyssey Infrared Imaging System (LI-COR) and scanned in the 700 *nm* channel with a 0.00 mm focus offset at 84 μm/pixel resolution.

***Electrophoresis and Western blotting***

To assess the quality of each protein preparation method, 5μg of protein from each sample preparation method were added to a 10% SDS-PAGE gel (Harlow and Lane 1988) and underwent electrophoresis at 125 VDC until the dye front approached the bottom of the gel. The contents of the gel were transferred to PVDF as per the manufacturer's instructions and total protein visualized by colloidal gold (BioRad). The resolution of protein bands was compared between each preparation method.

To test the utility of various commercial anti-*hsp* antibodies in assessing the *hsp* content in single polyp preparations, five identical SDS-PAGE gels (Harlow and Lane 1988) were loaded with pre-stained molecular weight markers (BioRad), and 10 ng of

*hsp*70, *hsp*90, and *hsc*70 (NSP-555, SPA-770 and SPA-751, Stressgen Bioreagents), to serve as positive controls and calibrants for gel quantification. The remaining wells were loaded with a volume equivalent to 5 µg of total protein from each coral polyp homogenate (computed from the quantification of total protein). The proteins were then electrophoresed at 125 VDC until the dye front reached the bottom of the gel. The contents of the gel were transferred to PVDF as per the manufacturer's instructions. Membranes were then blocked with 3% casein for one hour with shaking, and then incubated overnight at 4°C with 20 ml of a 0.5 µg/ml solution of an appropriate primary antibody washed three times with PBS, then incubated for one hour with the appropriate secondary antibody and washed again (*cf.* Table 1). The wet membranes were scanned on the Odyssey Infrared Imaging System (LI-COR) in both the 700 and 800 nm channels, with a 0.00 mm focus offset at 84 µm/pixel resolution.

**Table 1.** Details of the five positive control/calibrants for *hsps*, primary antibodies and fluorescent secondary antibodies, trialed in concert with the Odyssey Infrared Imaging System (LI-COR).

| | **Positive control/calibrants** | **Primary antibodies** | **Secondary antibodies** |
|---|---|---|---|
| i) | Human anti-*hsp*70 Protein (NSP-555 Stressgen) | Rabbit *hsp*70 (SPA-812 Stressgen) | Anti-Rabbit IR 680 (Molecular Probes) |
| ii) | Bovine *hsc*70 Protein (SPA-751 Stressgen) | Rat anti-*hsc*70 (SPA-815 Stressgen) | Anti-Rat IR800 (Jackson ImmunoRes) |
| iii) | Human *hsp*70 Protein (SPA-770 Stressgen) | Rat anti-*hsp*90 (SPA-840 Stressgen) | Anti-Rat IR800 (Jackson ImmunoRes) |
| iv) | Bovine *hsc*70 Protein (SPA-751 Stressgen) | Mouse anti-*hsc*70 (MA3-006) Biogen) | Anti-Mouse IR680 (Molecular Probes) |
| v) | Human *hsp*70 Protein (SPA-770 Stressgen) | Rabbit ant-*hsp*70 (En Virtue Biotech) | Anti-Mouse IR680 (Molecular Probes) |

***Total Protein and Heat Shock Protein Quantification***

The utility of the Odyssey Infrared Imaging System (LI-COR) for accurate assessment of both *hsp* expression via western blotting and total protein analysis using Coomassie blue staining was assessed. For protein estimation, a logarithmic dilution series of protein standards (BSA) (3.12, 6.25, 12.5, 25, 50, 100, 200, 400, 800, and 1600 μg/ml) were loaded onto PVDF and processed as described above. To assess the range of detection for western blotting, a series of *hsp* calibrant concentrations (10 pg – 100 ng) were loaded onto a single SDS-PAGE gel and assessed via western blot. Quantification of *hsps* in the polyp samples was achieved by comparing the scanned *hsp* intensity to the calibrant present on each gel. The calibrant for each gel was normalized to allow comparison of intensities between gels.

To assess if either the hsp70 or hsp90 antibodies would detect quantifiable differences in stress proteins, single coral polyps were assessed from laboratory control colonies maintained at 26.5° C in the coral microcosm at the Virginia Institute of Marine Science. Two single coral polyps were removed from each of the test coral colonies (n = 4). Thereafter, the same coral colonies were thermally shocked (+ 4° C ± 0.1) for 30 minutes, and single polyps (n = 8) were again removed from colonies (n = 4). Coral polyps (n = 16) (8 control, 8 heat shocked) were then processed for total protein using the method (v) described above, and western blots performed simultaneously using the *hsp*70 and *hsp*90 antibodies.

*Validation of Protocols*

To ensure the validity of the developed method and results, four control experiments (i-iv) were performed. To ensure the optimized protein recovery method was able to precipitate the targeted proteins *hsps*, (i) unstressed coral polyps (n = 3) were spiked with a known concentration (250 pg) of *hsp*70 calibrant and subjected to the sample handling procedures described above, and the polyps were then assayed for *hsp* content, pre- and post-labeling. To ensure that the relatively small amounts of coral mucus and seawater contained within each sample were also not contaminating sources of *hsps*, (ii and iii) the protein from 3 ml of mucus and 3 ml of seawater (n = 3) were precipitated using the new method described above. To validate that protein was not being discarded during the polyp preparation process (iv), 5 ml of pooled supernatant (n = 3) from the initial centrifugation, was then re-incubated overnight using method (v).

# RESULTS

## *Protein Concentration Estimation*

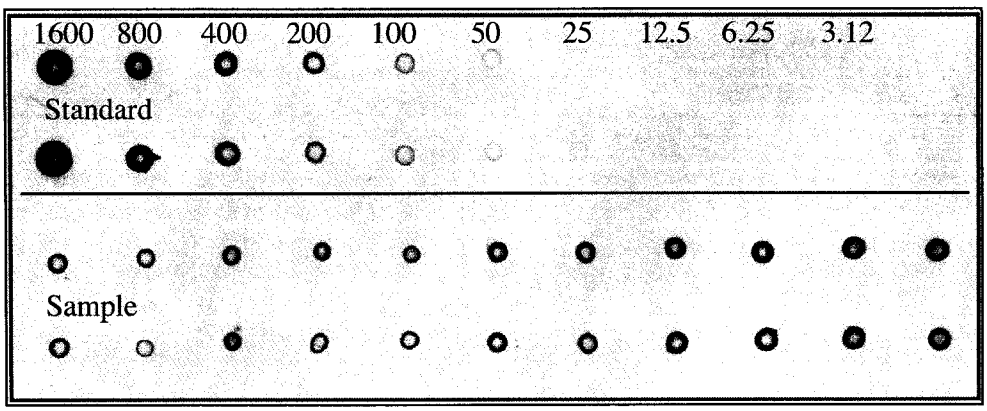
The described method for protein estimation using Coomassie blue staining, detection and quantification with the Odyssey Infrared Imaging System (LI-COR) demonstrated that a solution containing as little as 3.12 μg/ml of protein (6.24 ng total protein) could be reliably assessed. The relationship between protein concentration and scanned intensity was linear over the 2.5 log range of protein concentration (*cf.* Figure 3 A & B).

## *Polyp Preparations and Protein Recovery*

Each method was assessed in relation to time of preparation and yield of protein. The best method for both time and yield was method (v) (*cf.* Table 2) followed by method (iii). The method of Downs and others (2002) (i) resulted in a moderate recovery of protein but direct comparison of protein yield could not be achieved because of the differences in the initial polyp preparations, that is liquid nitrogen and sonication (Downs) vs. Laemmli sample buffer, with grinding and boiling (this study). The SDS-PAGE protein profiles of each method demonstrated that method (i) contained a high level of interfering contaminants as evidenced by smearing that inhibited resolution of the proteins (*cf.* Figure 4).

**Figure 3 A.** The scanned image of a Coomassie-stained PVDF blot of protein standards and coral polyp samples (in duplicate). Two µl of protein standards and polyp homogenates were pipetted onto PVDF and stained with Coomassie blue. The de-stained PVDF was allowed to dry, placed face down on the surface of the Odyssey Infrared Imaging System (LI-COR) and scanned in the 700 nm channel with a 0.00 mm focus offset at 84 µm/pixel resolution. Total protein concentration of the polyps were estimated against the protein standards. **B.** The relationship between scanned intensity and protein standards demonstrates the linear range of detection using Coomassie blue. *Error bars* are standard errors of the mean. Statistical analyses were performed using GraphPad Prism (version 4).

A

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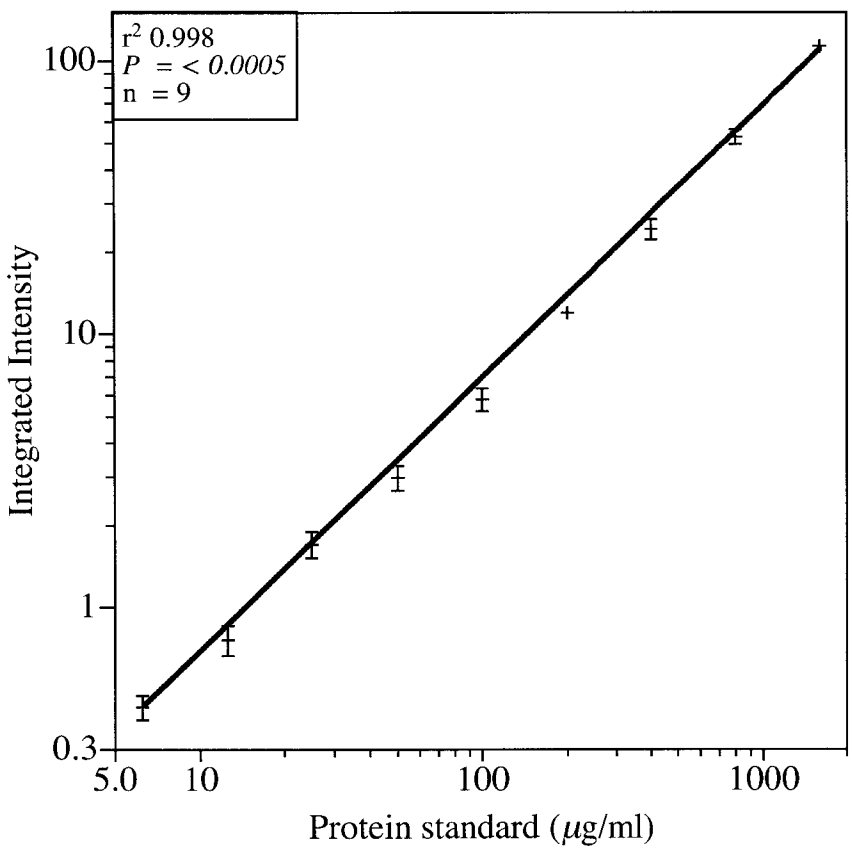
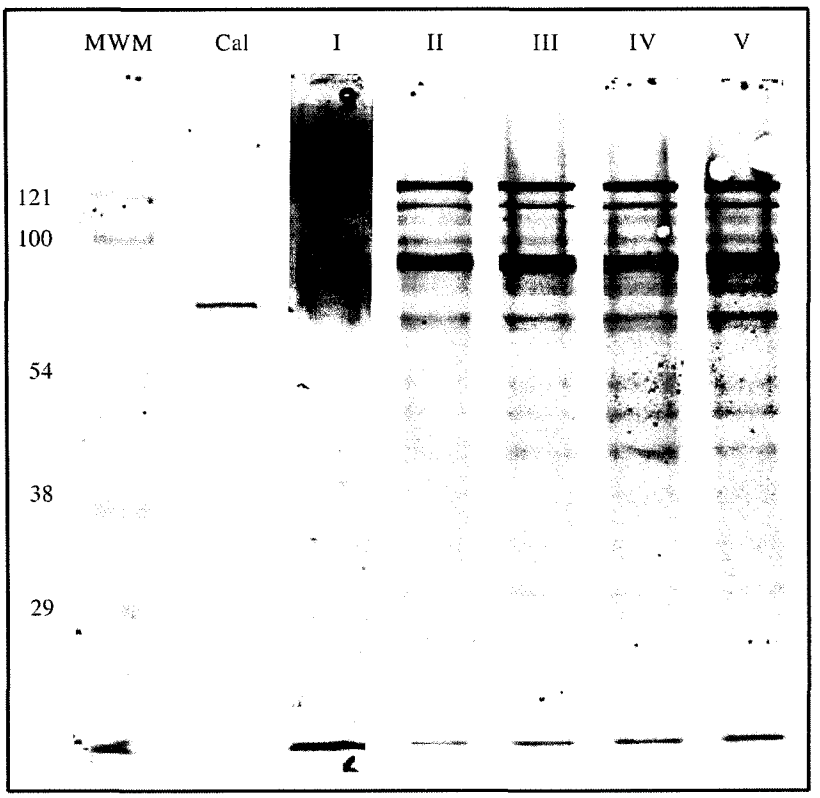


**Table 2.** Handling times and recovery of total protein from (n = 25) single coral polyps using a range of methods during processing. For yield, mean ± standard deviation reported.

| | **Method** | **Preparation time** | **Total protein yield (μg)/polyp** |
|---|---|---|---|
| i | Downs | 2 days | 67 +/- 15 |
| ii | Ammonia Sulfate | Overnight | 47 +/- 12 |
| iii | Acetone | Overnight | 62 +/- 6 |
| iv | Trichloroacetic acid + Deoxycholate (TCA + DOC) | Overnight | 56 +/- 13 |
| v | TCA + DOC + Acetone 2-mercaptoethanol | 5 hours | 77 +/- 9 |

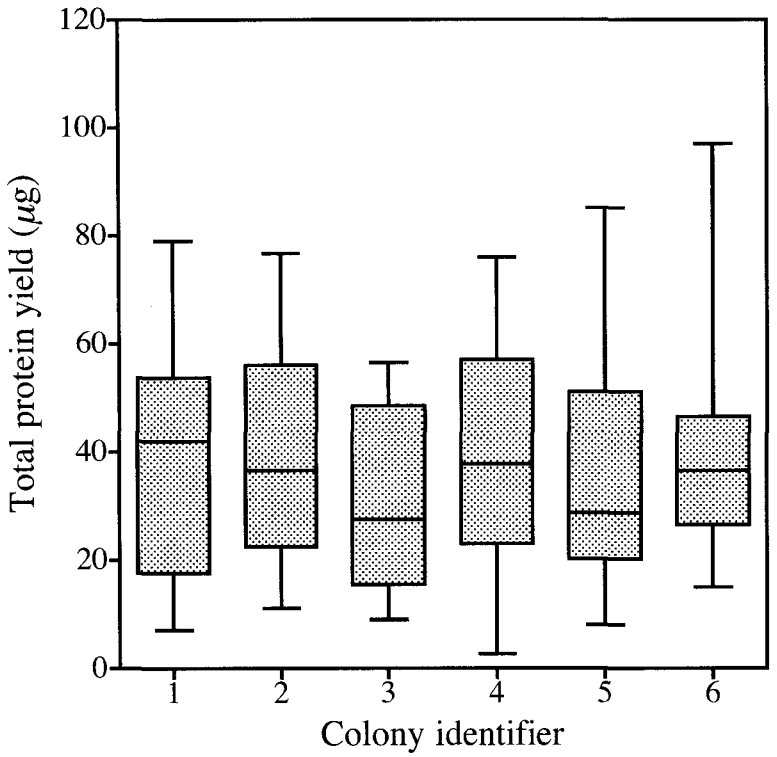
**Figure 4**. The colloidal gold protein profiles, of individual coral polyps processed by five different methods. Five µl of total protein was added to each lane (I - V) representing the polyp processing methods (i - v) from Table 2, respectively. Pre-stained broad range molecular weight markers (MWM) molecular weights are indicated on the left, adjacent to 100 ng of *hsp*70 calibrant (Cal).



The four other methods (i – iv) all displayed a similar protein resolution profile, with protein bands appearing sharp and in similar ratios between methods. Therefore, due to the similarity in total protein resolution, faster polyp preparation time and the increased total protein recovery, polyp preparation method (v) was used throughout.

The reliability of the polyp sampling method was confirmed after ~ 200 polyps were sampled from six individual coral colonies (~ 35 per colony), during two field experiments in 2002 and 2003 (*cf.* Chapters 3.). Polyps were processed by method (v), and the total protein recovery, per single coral polyp was determined (*cf.* Figure 5). The median recovery from each polyp, irrespective of sampled colony, was fairly homogeneous with only a few outliers.

**Figure 5**. Box plot with 25$^{th}$ percentile (lower hinge), median (bar) and 75$^{th}$ percentile (upper hinge) shown. Validation of single coral polyp sampling method, derived from the level of protein recovery. Total protein recovered using method (v), from individual coral polyps (n = 200) at ~30 polyps per coral colony from six colonies of *Montastrea annularis*.



***Antibody Assessment for Heat Shock Protein Quantification***

Polyp preparations purified by method (v) were used to assess a range of anti-*hsp*/*hsc* antibodies, used in prior coral *hsp* studies (*cf.* Table 1). Only the *hsp*70 and *hsp*90 (Stressgen) displayed consistent high-resolution reactivity with proteins at 70 and 90 kDa respectively in heat-shocked samples (*cf.* Figure 6). No reactivity was seen with the anti-*hsc*70 (Stressgen) or the anti-*hsp* 70 (Biogen), and no specificity was observed with the *hsp* antibody from Envirtue Biotechnology.

The dynamic range of *hsp* detection via western blotting using the Odyssey Infrared Imaging System (LI-COR) was determined to be from 5 pg to 100 ng (*cf.* Figure 7 A & B). Further, over the protein range assessed there was a linear relationship between protein concentration and scanned intensity. This relationship allows a single calibrant concentration to be added to the gels and used for *hsp* concentration estimation in samples.

Single coral polyps (n = 8) were sampled from colonies (n = 4) maintained at 26.5° C, and then again following a thermal shock treatment (+ 4° C ± 0.1 for 30 minutes) (n = 8), to assess if either the *hsp*70 or *hsp*90 antibodies would detect quantifiable differences in stress proteins. A *Student's t-test* resolved a significant difference in the expression of the *hsp*70 between the treatment groups ($p < 0.0001$), whilst no significance was observed with the *hsp*90 ($p > 0.5$) (*cf.* Figure 8).

**Figure 6.** The simultaneous detection of *hsp*70 and *hsp*90 demonstrated via western blotting, using the Odyssey Infrared Imaging System (LI-COR). Five μg of thermally shocked coral polyp homogenate was added to a lane of an SDS-PAGE gel and assessed by western blot, utilizing the 700 nm (red - *hsp*70) and the 800 nm (green - *hsp*90) channels of the Odyssey Infrared Imaging System (LI-COR). The single polyp lane demonstrates the expression of both *hsp* forms.

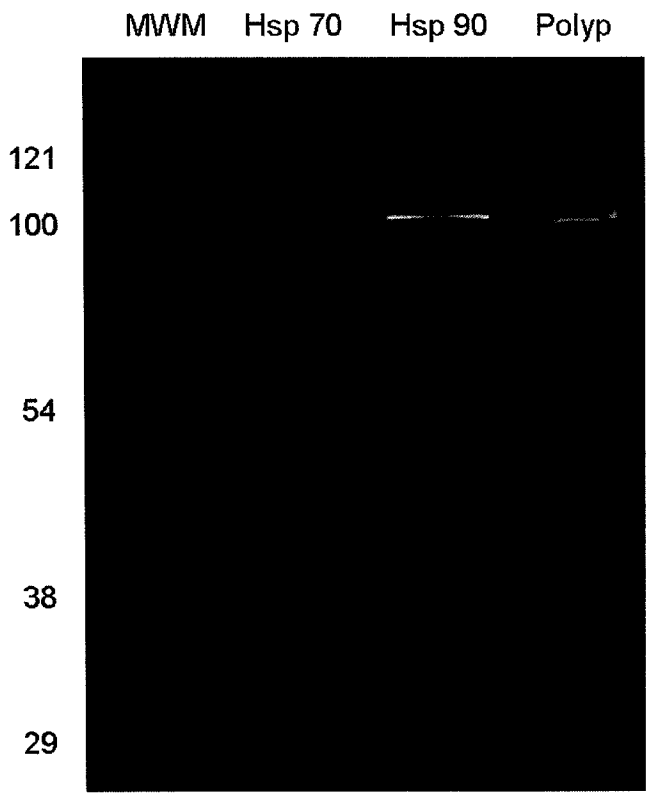
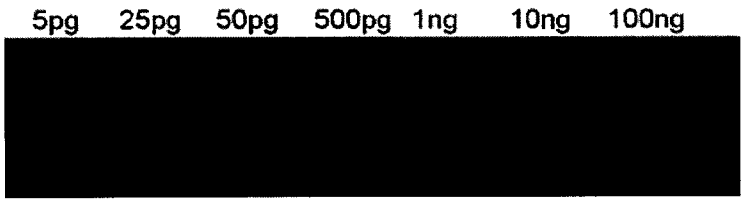


**Figure 7.** Relationship between the protein concentration of the *hsp*70 calibrant, and integrated intensity. **A.** A concentration series of *hsp*70 calibrant was loaded onto an SDS-PAGE gel and assessed via western blotting. **B.** There was a linear relationship between protein concentration and signal intensity. Statistical analyses were performed using GraphPad Prism (version 4).

A

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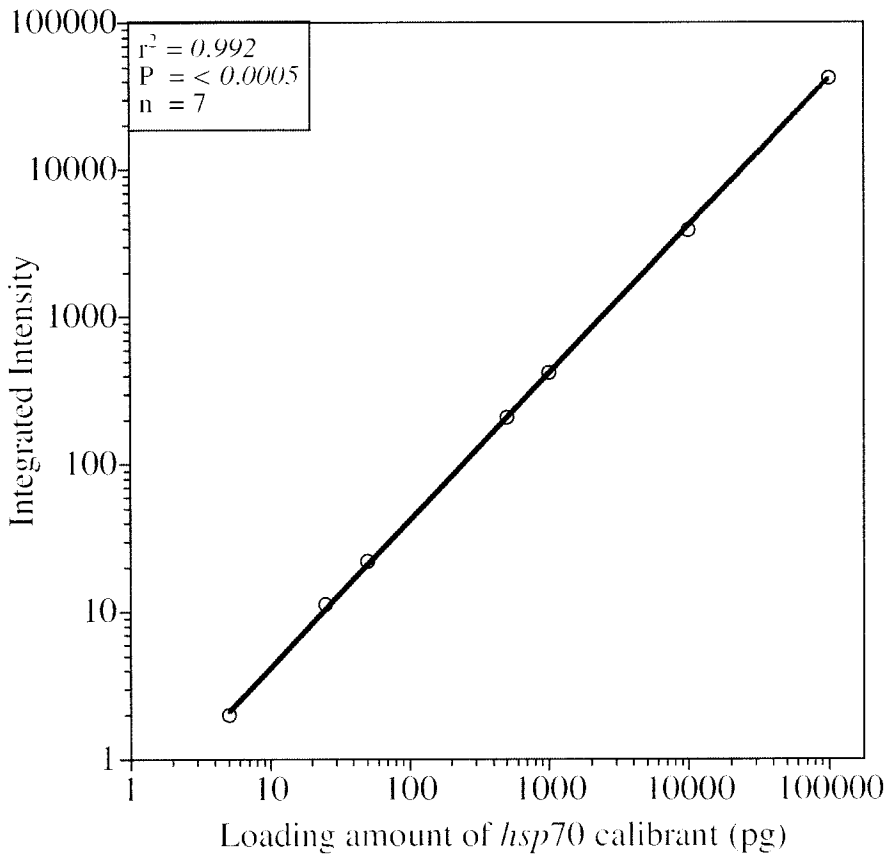
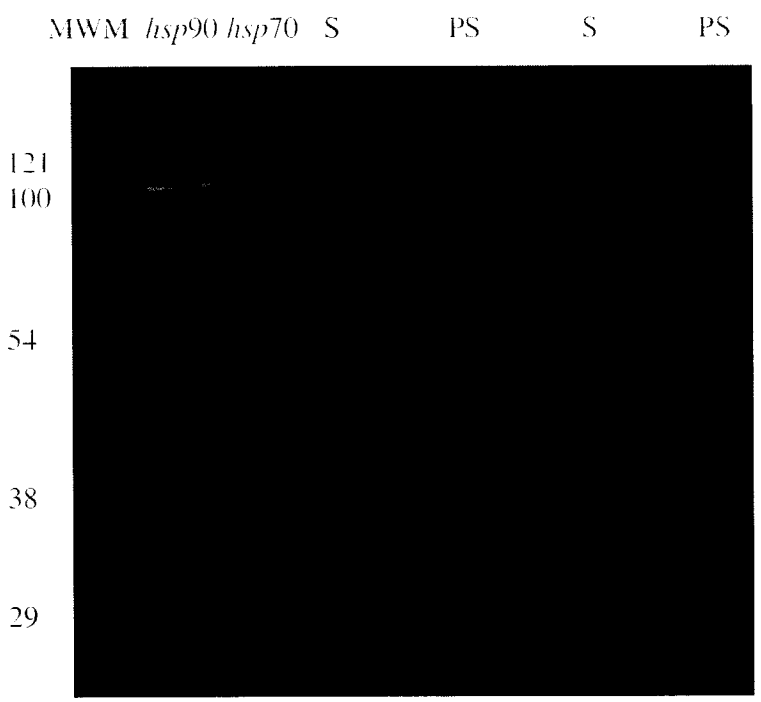


**Figure 8.** The expression of heat shock protein between thermally shocked (+ 4°C ± 0.1) (S) (n = 8), and pre-stressed coral polyp samples (PS) (n = 8). **A.** Coral colonies (n = 4) were subjected to thermal shock and the expression of *hsp*70 and *hsp*90 and measured via western blot. **B.** The polyps demonstrated an elevated expression of *hsp*70 in relation to heat stress, whilst no significant difference in expression was observed with *hsp* 90. *Error bar*s show standard error. Statistical analyses were performed using GraphPad Prism (version 4).

A

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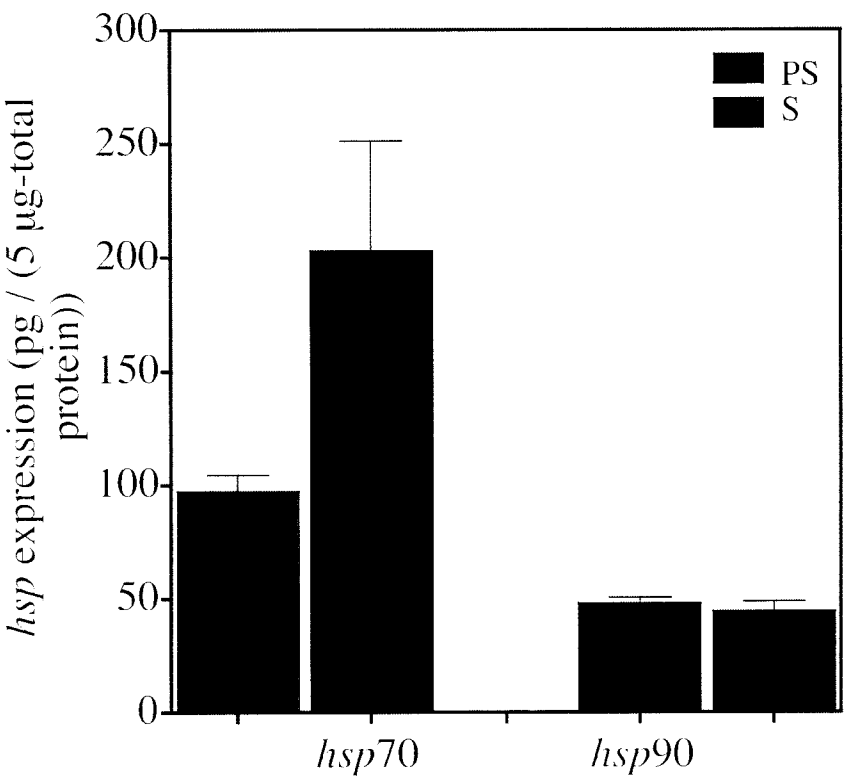


*Validation of Protocols*

Coral polyps (n = 5) were spiked with a known concentration of *hsp*70 calibrant. Method (v) resulted in a mean recovery of 93.7 +/- 3.4 %. No detectable protein content was observed in the coral mucus or seawater associated with each coral sample, or the supernatant from the initial centrifugation at the polyp preparation stage. Therefore, their combined contribution to the quantifiable *hsp* concentration must be below the detection limits used.

# DISCUSSION

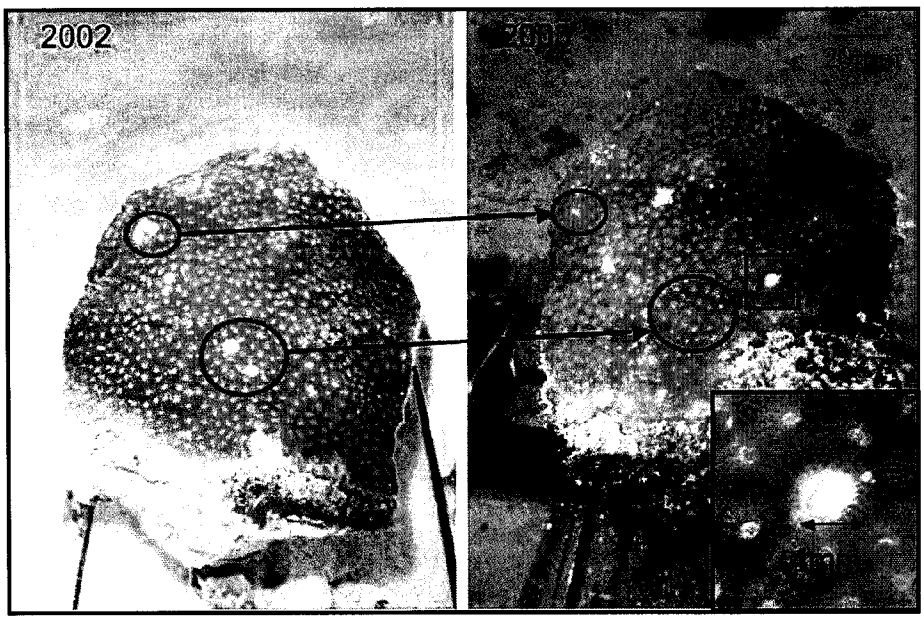
The study of stress protein expression in corals has been subject to many limitations, including potentially destructive sampling methods, and excessive sample handling and preparation, resulting in protein degradation and poor western blot resolution. This study introduces novel methods for sample acquisition, polyp processing and quantification that resolves many of these problems. The method described could potentially be applied to a range of coral studies and management issues.

Current collection methods can leave coral reefs damaged and open to disease and additional stress. This damage often results in laboratory processing methods having to account for $CaCO_3$ and coral mucus contamination. Polyp by polyp sampling also known as micro-tissue collection techniques were previously employed to collect coral tissue for lipid analysis, using a Vacutainer blood collection tube (10 ml) and a 20 x 2.0 mm gage Vacutainer blood collection needle (Niebuhr 1999). The needle was inserted into individual polyps and rotated around the interior of the calyx to loosen the polyp tissue prior to collection. After the tissue was detached, the Vacutainer tube was placed on the needle, and the tissues were transferred to the Vacutainer tube. This procedure was carried out in a controlled laboratory setting following the removal of the coral samples from the reef. This sampling technique carries the disadvantage of the Vacutainer needle becoming blocked with carbonate debris and given the dexterity required, was not

considered appropriate for divers to utilize at 60 foot depth whilst saturated. For coral studies that require low sample volume, or non-destructive time series data collections, the sampling method presented here has many advantages, for example reduced damage to the underlying $CaCO_3$ skeleton and the lack of mechanical shock from hammer blows. This method has already been demonstrated in the successful collection of non-destructive time series data from small coral colonies over two field seasons (*cf.* Chapter 3). This method can be used equally well in a laboratory setting, carried out by SCUBA divers or during shallow water snorkelling. Additionally, coral reef systems can now be routinely sampled whilst monitoring pre- and post- events of interest. The utility of this method should have dramatic impacts in the molecular study of coral reef systems.

Researchers should note the potential ecological advantages of this study, as typical studies routinely utilize ~ 100 $cm^2$ of coral colony per sample. Localized damage to the colony resulting from the non-surgical excision of tissue for example with hammer and chisel, may lead to infection or further degradation of adjacent fragmented coral. In contrast, our method utilized *ca.* 12 $mm^3$ of coral tissue per sample and all corals (n = 6) survived for more than two years following commencement of polyp sampling. Complete recovery of excised polyps sites within all corals (n = 6) was noted at the beginning of the second field season (2003) and in 2004 when corals where retrieved from study site (*cf.* Figure 9). Coral recovery appeared in keeping with the current understanding of coral regeneration (Lea-Anne Henry 2005; Titlyanov and others 2005). If small sample volumes are appropriate, researchers no longer have to destructively sample the species they wish to study.

**Figure 9.** Coral # 6 photographed in 2002 and 2003. Re-growth of polyps appeared in keeping with the current understanding of coral regeneration (Lea-Anne Henry 2005; Titlyanov and others 2005). Inset shows a typical 4 mm single polyp excision wound.



The ability of researchers to acquire numerous replicates from a single colony through repeated sampling over time would generate robust statistical analysis leading to more detailed studies and effective management decisions.

In order to accurately assess the recovery of total protein from the sampling method described, a protein quantification method was developed that was highly sensitive, rapid and reliable. Since the Coomassie blue protein stain fluoresces in the near infrared, it can be readily detected with the Odyssey Infrared Imaging System (LI-COR) 700 nm laser. Thus a protein quantification assay was developed loosely-based on a published method (Ghosh and others 1988).

This method provides a rapid and accurate approach of quantifying protein concentration of samples even in the presence of substances that would normally interfere with protein estimation, for example SDS. Further, the method only required 4 µl of sample (2 x 2 µl duplicates), had a sensitivity of ~ 3.12 µg/ml, had a wide linear range of 2.5 orders of magnitude, and quantification of up to 20 samples could be achieved in less than five hours. The main limitation of this method is that samples need to be loaded within 10 minutes onto the PVDF membrane before membrane dries and becomes inactive for protein binding.

Utilizing a method employed by previous studies for coral polyp preparation (Brown and others 2002; Downs and others 2000), we were unable to gain the sensitivity and resolution of proteins required to study *hsp* expression in single coral polyps.

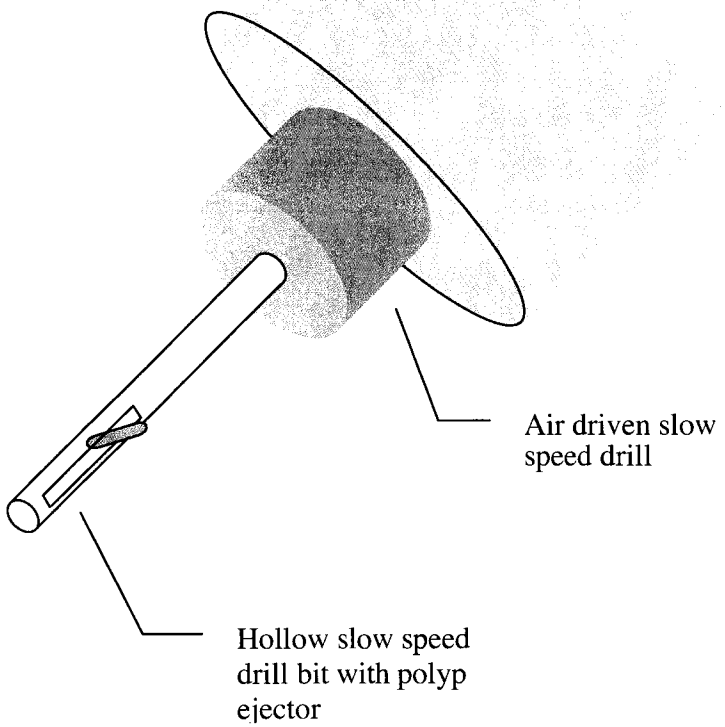
Therefore we researched alternate methods that would allow reliable recovery of proteins from the contaminating substances such as lipids and polysaccharides. A recently described method utilized acetone precipitation to study *hsps* from 1 - 3 $cm^2$ pieces of *Agarcia agaricites* (Linnaeus 1758) and *Agarcia tenufolia* (Dana 1848) (Robbart and others 2004; Robbart 2002). Building upon this previous work, other methods where examined that utilized protein precipitation, especially those used in plant protein analysis, which are subject to poor SDS-PAGE resolution when contaminants are present (Carpentier and others 2005; Saravanan and Rose 2004). Method (v) is similar to the methods widely used in plant protein analysis and protein preparation for 2D-SDS-PAGE, and allows the simultaneous precipitation of protein, while retaining lipids and polysaccharides in the fluid phase for extraction. Subsequent acetone washes of the precipitated protein removed contaminants that were caught within the protein during the precipitation process. This simplified sample preparative method allowed for high protein recovery (94%), and improved resolution in SDS-PAGE gels (*cf.* Figure 6 & 8 A).

Following sample preparation we were able to resolve *hsp* expression utilizing traditional chromagen deposition-based methods such as aminoethylcarbazol and alkaline phosphatase. The limited sensitivity and range of detection of these methods, however, precludes accurate densitometric analysis. The Odyssey Infrared Imaging System (LI-COR) is a new imaging system that utilizes fluorescent dyes in the 700 and 800 nm range, allowing dual detection of proteins. This detection system was able to assess both *hsp*70 and *hsp* 90 expressions in a single sample. The wide linear range of this system

(5pg - 50ng) allowed quantification of *hsp*70 from 8.75 to 772 pg per 5 μ*g* of coral protein.

The polyp-spike is not presented as a panacea to coral sampling, but more a working prototype that would benefit from further development. Retaining the excised polyp in the tip of the spike, its subsequent removal, and maintaining a consistent sample-size throughout the study, was routinely achieved, although challenging. A natural evolution of the polyp-spike would be an pneumatic slow-speed drill, with a polyp size-specific hollow bit, thus ensuring uniformity in soft tissue sampling and rendering the excised polyp effectively captured until transferred (*cf.* Figure 10). When purifying such small volumes (50 - 100 μl), sample loss, and cross-contamination are potential issues. Care should be exercised when using glass or Teflon Eppendorf-pestles, as these can retain protein when removed from the sample. Caution should also be taken when drying the membrane on the Whatman filter paper and in the pipetting of the protein samples. If the PVDF is too wet samples will spread, potentially contaminating adjacent areas. Allowing the PVDF to dry too soon during the pipetting phase may cause failure in the binding of the protein. These trials indicated in the region of 20 sample replicates and protein series to be the most efficient number to process on a single membrane.

**Figure 10.** A diagrammatic representation of a proposed pneumatic driven, slow-speed drill with a polyp size-specific (hollow) bit to ensure uniformity in soft tissue sampling whilst rendering the excised polyp captured until transferred.



*Summary*

Coral reefs form the basis of a wide range of studies. As the detection limits of instrumentation improves, it is important that attention be focused on how samples are collected, as these data (and its acquisition) are integral to the validity of any research and should not therefore be restrained by employing only established methods. Given the small volume and relatively small amounts of recoverable protein from individual polyps, these novel methods may not necessarily be appropriate for all applications. Nonetheless, they proffer the wherewithal for future coral studies to reduce the size of coral sampled from, for example, a cricket ball down to a tiddledy-wink and at the same time, inflict less damage, reduce the amount of sampling stress, and allow for extended studies within the same coral colony.

## LITERATURE CITED

Achituv Y, Ben-Zion M, Mizrahi L. 1994. Carbohydrate, lipid, and protein composition of zooxanthellae and animal fractions of the coral Pocillopora damicornis exposed to ammonium enrichment. Pacific Science 48(3):224-233.

Ben-Haim Y, Thompson FL, Thompson CC, Cnockaert MC, Hoste B, Swings J, Rosenberg E. 2003. *Vibrio coralliilyticus* sp. nov., a temperature-dependent pathogen of the coral *Pocillopora damicornis*. International Journal of Systematics Evolution and Microbiology 53:309-315.

Black NA, Voellmy R, Szmant AM. 1995. Heat shock protein induction in *Montastraea faveolata* and *Aiptasia pallida* exposed to elevated temperatures. Biological Bulletin 188:234-240.

Brown BE, Downs CA, Dunne RP, Gibb SW. 2002. Exploring the basis of thermotolerance in the reef coral *Goniastrea aspera*. Marine Ecology Progress Series 242:119-129.

Buckley BA, Hofmann GE. 2002. Thermal acclimation changes DNA-binding activity of heat shock factor 1 (HSF1) in the goby Gillichthys mirabilis: implications for plasticity in the heat-shock response in natural populations. J Exp Biol 205(20):3231-3240.

Bythell JC, Sharp VA, Miller D, Brown BE. 1995. A novel environmentally-regulated 33 kDa protein from tropical and temperate cnidarian zooxanthellae. Journal of Thermal Biology 20(1/2):15-22.

Carpentier SC, Witters E, Laukens K, Deckers P, Swennen R, Panis B. 2005. Preparation of protein extracts from recalcitrant plant tissues: an evaluation of different methods for two-dimensional gel electrophoresis analysis. Proteomics 5(10):2497-507.

Cox EF, Lewis TL. 2003. Molecular Biology of Corals. University of Hawai'i Hawai'i Institute of Marine Biology 107 p.

Downs CA, Fauth JE, Halas JC, Dustan P, Bemiss J, Woodley CM. 2002. Oxidative stress and seasonal coral bleaching. Journal of Free Radical Biology and Medicine 33(4):533-543.

Downs CA, Mueller E, Phillips S, Fauth JE, Woodley CM. 2000. A molecular biomarker system for assessing the health of coral (*Montastraea faveolata*) during heat stress. Marine Biotechnology 2:533-544.

Ducklow HW, Mitchell R. 1979. Composition of mucus released by coral reef coelenterates. Limnol Oceanogr 24(4):706-714.

Gardner TA, Côte IM, Gill JA, Grant A, Watkinson AR. 2003. Long-term region-wide declines in Caribbean corals. Science 301:958–960.

Ghosh S, Gepstein S, Heikkila JJ, Dumbroff EB. 1988. Use of a scanning densitometer or an ELISA plate reader for measurement of nanogram amounts of protein in crude extracts from biological tissues. Analytical Biochemistry 169:227-233.

Griffin SP. 2005. Comparison of Molecular Biomarkers within and across Scleractinian Coral Species exposed to Elevated Temperatures. Puerto Rico: University of Puerto Rico. 139 p.

Griffin SP, Bhagooli R. 2004. Measuring antioxidant potential in corals using the FRAP assay. Journal of Experimental Marine Biology and Ecology 302:201 - 211.

Harlow E, Lane D. 1988. Appendix 1: Electrophoresis. Antibodies a laboratory manual: Cold Springs Harbor Laboratory. p 635-59.

Hoegh-Guldberg O. 1999. Climate change, coral bleaching and the future of the world's coral reefs. Marine and Freshwater Research 50:839-866.

Hofmann GE. 2005. Patterns of Hsp gene expression in ectothermic marine organisms on small to large biogeographic scales. Integrated Comparative Biology 45(2):247-255.

Hughes TP, Baird AH, Bellwod DR, Card M, Connolly SR, Folke C, Grosberg R, Hoegh-Guldberg O, Jackson JBC, Kleypas J and others. 2003. Climate change, human impacts and the resilience of coral reefs. Science 310:929-933.

Jackson JBC, Kirby MX, Berger WH, Bjorndal KA, Botsford LW, Bourque BJ, Bradbury RH, Cooke R, Erlandson J, Estes JA and others. 2001. Historical overfishing and the recent collapse of coastal ecosystems. Science 293:629-637.

Knowlton N. 2001. The future of coral reefs. Proceedings of the National Academy of Sciences 98(10):5419-5425.

Krupp DA. 1985. An immunochemical study of the mucus from the solitary coral Fungia scutaria (Scleractinia, Fungiidae). Bulletin of Marine Science 36(1):163-176.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lea-Anne Henry MH. 2005. Regeneration from Injury and Resource Allocation in Sponges and Corals - a Review. International Review of Hydrobiology 90(2):125-158.

Miller D, Brown BE, Sharp VA, Nganro N. 1992. Changes in the expression of soluble proteins extracted from the symbiotic anemone Anemonia viridis accompany bleaching induced by hyperthermia and metal stressors. Journal of Thermal Biology 17(217-223).

Miller. K J, Ayre DJ. 2004. The role of sexual and asexual reproduction in structuring high latitude populations of the reef coral *Pocillopora damicornis*. Heredity 92(6):557-568.

Niebuhr DH. 1999. Environmental Stress in Hard Coral: Evaluating Lipid as an Indicator of Sub-lethal Stress on Short Time Scales [Ph.D]. Williamsburg: College of William and Mary. 152 p.

Nystrom M, Folke C, Moberg F. 2000. Coral reef disturbance and resilience in a human-dominated environment. Trends in Ecology and Evolution 15:413-417.

Oku H, Yamashiro H, Onaga K, Sakai K, Iwasaki H. 2003. Seasonal changes in the content and composition of lipids in the coral Goniastrea aspera. Coral reefs 22(1):83-85.

Pandolfi JM, Bradbury RH, Sala E, Hughes TP, Bjorndal KA, Cooke RG, McArdle D, McClenachan L, Newman MJH, Paredes G and others. 2003. Global trajectories of the long-term decline of coral reef ecosystems. Science 301:955-958.

Robbart M, L., Peckol P, Scordilis S, P., Curran H, A., Brown-Saracino J. 2004. Population recovery and differential heat shock protein expression for the corals Agaricia agaricites and A. tenuifolia in Belize. Marine Ecology Progress Series 283:151-160.

Robbart ML. 2002. Population dynamics and differential heat shock protein expression in two Caribbean scleractinian corals, Agarica Agaricites and A, Tenuifolia [Masters]: Smith College. 98 p.

Rossi S, Snyder MJ. 2001. Competition for space among sessile marine invertebrates: changes in HSP70 expression in two Pacific cnidarians. Biol Bull 201(3):385-93.

Rowan R, Knowlton N, Baker A, Jara J. 1997. Landscape ecology of algal symbionts creates variation in episodes of coral bleaching. Nature (London) 388(6639):265-269.

Saravanan RS, Rose JK. 2004. A critical evaluation of sample extraction techniques for enhanced proteomic analysis of recalcitrant plant tissues. Proteomics 4(9):2522-32.

Sharp VA, Miller D, Bythell JC, Brown BE. 1994. Expression of low molecular weight HSP 70 related polypeptides from the symbiotic sea anemone *Anemonia viridis* Forskall in response to heat shock. Journal of Experimental Marine Biology and Ecology 179:179-193.

Titlyanov EA, Titlyanova TV, Yakovleva IM, Nakano Y, Bhagooli R. 2005. Regeneration of artificial injuries on scleractinian corals and coral/algal competition for newly formed substrate. Journal of Experimental Marine Biology and Ecology 323(1):27-42.

Toller WW, Rowan R, Knowlton N. 2001. Zooxanthellae of the *Montastrea annularis* species complex: Patterns of distribution of four taxa of *Symbiodinium* on different reefs and across depths. Biological Bulletin 201:348-359.

Wilkinson C, Linden O, Cesar H, Hodgson G, Rubens J, Strong AE. 1999. Ecological and socioeconomic impacts of 1998 coral mortality in the Indian Ocean: an ENSO impact and a warning of future change? Ambio - A Journal of the Human Environment 28:188-196.

Yamashiro H, Oku H, Higa H, Chinen I, Sakai K. 1999. Composition of lipids, fatty acids and sterols in Okinawan corals. Comparative Biochemistry and Physiology, B 122B(4):397-407.

## CHAPTER 3

# WATER FLOW INFLUENCES THE SPATIOTEMPORAL DISTRIBUTION OF HEAT SHOCK PROTEIN 70 WITHIN COLONIES OF THE CORAL *MONTASTREA ANNULARIS* (ELLIS AND SOLANDER, 1786): IMPLICATIONS FOR CORAL BLEACHING

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## ABSTRACT

Water-flow may perform a number of roles during and following episodes of coral reef bleaching. Increased water flow has the potential to develop the capacity of some scleractinian corals to cope with the elevated concentrations of oxygen in the boundary layer that are photosynthetically derived, whereas bleaching is often elevated in areas where water flow is restricted. On the other hand, water motion has been shown to enhance the metabolic rate both for photosynthesis and respiration, and such flow-modulated metabolism has the potential to create asymmetric bleaching patterns within a coral colony, because of the dissimilar effects of flow on these metabolic processes.

To broaden these earlier studies, we examined the effects of flow on constitutive and regulated stress proteins, we tested whether corals would exhibit a spatially asymmetric distribution of heat shock protein 70 and 90 (*hsp*70 and *hsp*90) and the constitutive stress protein 70 (*hsc*70) related to velocity gradients (degree of mixing) across the coral colony. Water-flow manipulations were conducted from the NOAA underwater habitat Aquarius on colonies of *Montastrea annularis* (Ellis and Solander, 1786) with controlled exposures to increased flow (ca. 40 cm $s^{-1}$) and increased temperature (*ca.* 1.5 - 2 $^{0}$C above ambient) using *in situ* flow chambers. Single coral polyps were sampled, processed and analyzed through a combination of novel protein recovery and quantification methods, along with standard sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting methods. The short term (daily) and medium term (nine days) response within discrete locations of the coral colonies were examined. *Montastrea annularis* colonies developed and sustained significant spatially asymmetric patterns of stress protein synthesis across the entire coral surface, with upstream sectors expressing more *hsp*70, at the same time that these upstream sectors developed and sustained a reduced Quantum Yield (QY) (*cf.* Chapter 1). The mechanism producing this pattern is unclear; we speculate that increased flow may lead to an initial up-regulation within the synthesis of heat shock protein by the entire colony, followed by a down-regulation in discrete areas through increased hydraulic stress or biochemical energy requirements and limitations.

## INTRODUCTION

The majority of reef-building scleractinian corals live within a few degrees of their upper thermal limits and often experience heat stress following temperature increases of only 1.5 – 2° C (Fitt and others 2001; Jokiel 1977). Coral bleaching has been linked with thermal excursions that occur with elevated sea-surface temperatures (SST) often in concert with El Niño-Southern Oscillation (ENSO) events (Brown 1997; Glynn 1988; Wilkinson 2000). Bleaching has been investigated in laboratory heat shock experiments, for example (Hayes and King 1995), and long-term studies of coral reefs affected by thermal anomalies followed by coral mortality have demonstrated that coral recovery may take several decades (Glynn 1984; Robbart 2002).

There are several natural and anthropogenic triggers of coral stress; *e.g.*, thermal extremes (Brown 1997; Brown and others 2002b; Buddemeier and Fautin 1993; Jones and others 1998; Saxby and others 2003), light saturation (Banaszak and Trench 1995; Brown 1997; Jokiel and Coles 1974), bacterial infection (Ben-Haim and others 1999; Ben-Haim and others 2003; Hughes 1994; Richardson and others 1998), sedimentation (Brown 1997; Cole 2003), and high irradiance including UV (Gleason and Wellington 1993), all of which are associated with a host of deleterious impacts on corals (Brown 1997; Fitt and Warner 1995; West and Salm 2003). Coral stress can often occur when the regular operating limits of the aforementioned triggers drift outside the corals' narrow

range of optimum environmental conditions. In many cases these environmental excursions can initiate the breakdown of the coral's metabolic functions and culminate in the expulsion of the coral's algal symbiont and/or degradation of the symbiont's photosynthetic pigments (Brown 1997; Brown and others 2002a; Downs and others 2002). Following the loss of symbiont and/or pigment the coral colonies can often appear white or bleached (Brown 1997; Glynn 1993; Hoegh-Guldberg 1999). Bleaching events are often accompanied by (but not restricted to) a reduction in reproductive effort, an increased susceptibility to disease, and a loss of healthy coral, including mortality within a range of temporal and spatial scales (Brown 1997; Brown and others 2002a).

***Water Flow and Coral Stress***

Seemingly benign environments often harbor microhabitats capable of eliciting mild episodes of stress that if sustained can accumulate, and incite an up-regulation in the synthesis of stress proteins (Hochachka and Somero 2002). For example, weather conditions of little or no wind, clear skies and increased air temperatures, which might appear innocuous in the short term, if sustained, can illicit a range of stressful responses within coral reefs systems.

Increased irradiance and sea surface temperatures are often associated with low water-flow (hot and calm weather) and therefore reduced mass transport. These particular conditions are often coincident with high rates of photosynthesis that can cause levels of reactive oxygen species (ROS) to build within the soft tissue of corals (Downs and others 2002; Jones and others 1998; Lesser 1997; Lesser 2006).

Overlaying these conditions with, instead, a sustained high water-flow may further challenge the organism and restrict its ability to carry out essential metabolic processes, such as the regulation of stress protein synthesis. Flow-modulated metabolism has already been investigated within the respiration of octocorals and sea anemones (Patterson and Sebens 1989), as has flow-sensitivity of *M. annularis* within processes of respiration and photosynthesis (Patterson and others 1991). Patterson and Sebens (1989) cautioned that when comparing measurements made in an enclosed chamber to those in the field, chamber-induced flow artifacts (*e.g.*, blockage effects) required close consideration. Some of the pervasive problems associated with these experimental chamber designs are the acceleration of flow between the organism and chamber wall and the unidirectional nature of the chamber flow, compared to the bi-directional nature of many shallower habitats (Patterson and Sebens 1989). These issues applied to this study and were accounted for by a size restriction on all coral treatments, which would maximize the free-space between coral and chamber wall, in tandem with video analyses to resolve water-flow behavior at the coral-polyp level. Notwithstanding these potentially confounding artifacts, Patterson and Sebens's (1989) findings were in qualitative agreement with a range of previous studies. In all cases, enhanced flow speed increased the rate of gas exchange, and this process occurred at the coral-polyp level (Patterson and Sebens 1989). Whether the rates of gas exchange scale with increasing water-flow speed at the reef or landscape level still requires attention.

***Studies on the Advantages of Increased Water flow***

Samples of the scleractinian coral *Stylophora pistillata* (Esper 1797) were experimentally bleached and their recovery rates appeared to change in accordance with the rate of water-flow over the coral treatments (Nakamura and others 2003). During a seven-week manipulation, the number of zooxanthellae per $cm^2$ and chlorophyll *a* concentration ($\mu g\ cm^2$) in moderate flow conditions of *ca.* 20 cm $s^{-1}$ underwent a rapid recovery following an initial three weeks of stasis (Nakamura and others 2003). Coral colonies subjected to low-flow treatments ($\leq$ 3 cm $s^{-1}$) revealed only slight change in their condition. The authors went on to state that moderate to high water-flow rates might facilitate partial recovery from a bleaching event. It is also suggested that the mechanisms that lead to the dysfunction of the algal-coral symbiosis in the first instance, may be driven by a mass-transfer limiting process (Nakamura and van Woesik 2001).

The effect of a sustained increase in metabolic rates from increased water flow warrants further investigation. One line of enquiry is that fast-flowing water may only act as a short-term respite for bleaching stress. Following sustained periods of fast-flow, irradiance and thermal stress, a yet to be determined physiological threshold may trigger an over-burdening of the organism during the accompanying periods of high photosynthesis, including increased energy expenditure required for metabolic processes, such as the synthesis of molecular chaperones.

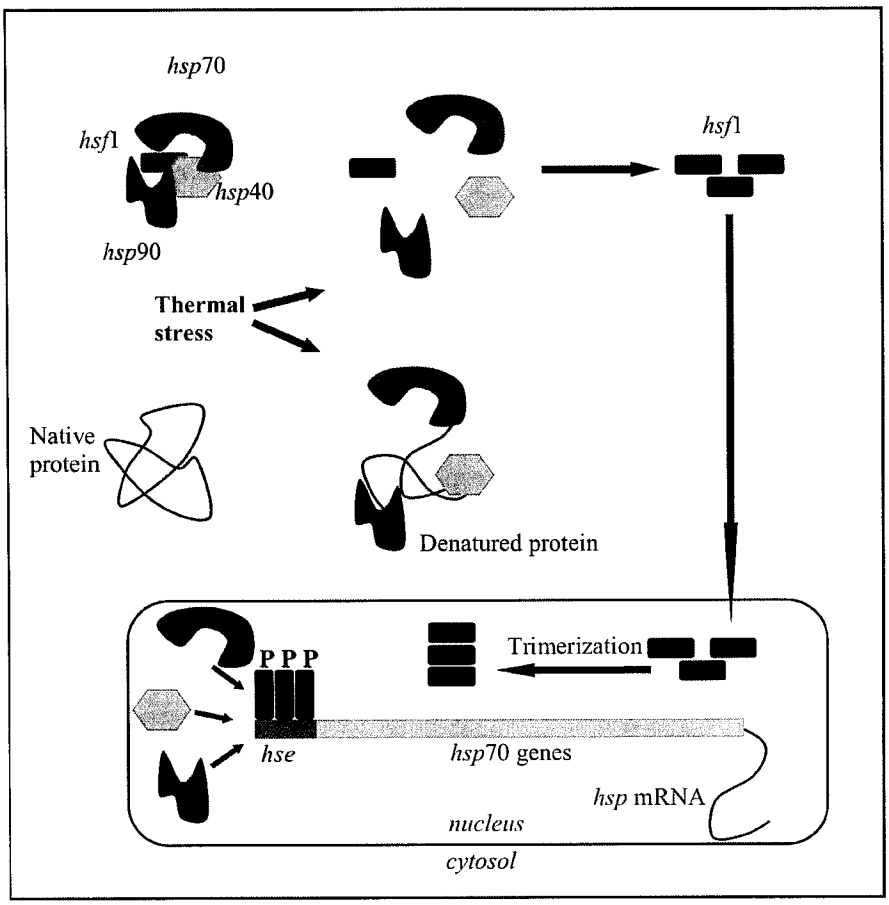
***Regulation of hsp70***

The regulation of heat shock factor 1 (*hsf*1) activity is complex and controlled (in part) by the fundamental properties of the molecule, specifically by thermally induced changes to the molecule's conformation. Inactive monomers are then stimulated to assemble into trimers that bind to the heat-shock element (*hse*), a gene regulatory element found upstream of most hsp-encoding genes. These studies suggested that *hsf*1 *detects* a thermal stress and may therefore be a type of *cellular thermometer*. Several studies, however, have demonstrated the temperature at which *hsf*1 is activated is variable, and implies that extrinsic factors may influence the regulation of *hsf*1 activity. These factors include *hsp*40, 70 and 90, which directly interact with *hsf*1 in a multi-protein complex under *in vivo* conditions (Hochachka and Somero 2002; Lindquist 1988; Tomanek 2002; Tomanek and Somero 2002).

It has been suggested the presence of these *hsps* ensure *hsf*1 is bound into a multi-protein complex whilst under non-stressful conditions thus preventing *hsf*1 from binding to the heat shock element and initiating the transcription of *hsp* genes. When environmental or physiological stresses occur that cause proteins to unfold, this *hsp*40-70-90/ *hsf*1 complex dissociates as theses *hsps* will preferentially bind to unfolded proteins rather than to *hsf*1. Heat shock factor 1 monomers are then able to relocate from the cytosol to the nucleus, trimerize, and bind to the *hse*. The trimers of *hsf*1 that are transcriptionally competent when bound to the *hse* are hyperphosphorylated, to render *hsf*1 transcriptionally active. Additional unknown structural transitions are required. These events are in general reversed during the attenuation of a heat shock response, with

additional proteins involved in a regulatory role. Within this regulatory scheme, which is termed the *cellular thermometer* model of *hsp* expression, *hsps* carry out an auto-regulatory role by controlling their own synthesis by governing the levels of free *hsf*1 within a cell. Therefore, during conditions within which endogenous levels of *hsps* increase, for example, in response to acclimation to thermal increase, the formation of the *hsp*40-70-90/ *hsf*1 complex is preferential, and results in a higher temperature at which heat shock gene expression is induced ($T_{on}$) for the synthesis of *hsps*. Regardless of the fact that *hsf*1 transcription is not induced by thermal stress and *hsf*1's activity is regulated post-translationally, the *cellular thermometer* model predicts that higher concentrations of *hsf*1 would, assuming equal levels of *hsp*70 and *hsp*90, favor lower $T_{on}$ values for *hsp* gene transcription due to the presence of increased amounts of free *hsf*1 for binding to the *hse* (*cf* Figure 1) (Hochachka and Somero 2002; Tomanek 2002; Tomanek and Somero 2002).

**Figure 1.** The regulatory model of transcriptional activation, initiating the *de novo* synthesis of heat-shock proteins (*hsps*). Under stress-free conditions, heat shock factor 1 (*hsf*1) monomers are associated with a chaperone complex that contains at least *hsp*70, *hsp*90 and *hsp*40. During thermal stress, the complex dissociates and binds to denatured proteins. Dissociation of the complex frees *hsf*1 monomers, which then move into the nucleus and bind to the heat shock element (*hse*). Heat shock factor 1 trimers bound to the *hse* become hyperphosphorylated (P) prior to becoming transcriptionally competent. As *hsp* levels increase, their binding to *hsf*1 triggers its dissociation from the *hse*, leading to a down regulation in *hsp* gene transcription (Adapted after Tomenak and Somero).



### *Heat Shock Proteins Specific to this Study*

As discussed in the general introduction, a range of stress proteins have already been documented in a cnidarians, such as the inducible forms of *hsp*70, *hsp*60 and *hsp*25, and interpreted as bio-indicators during and following increased thermal and UV radiation stress (Branton and others 1999; Downs and others 2000; Fang and others 1997; Hayes and King 1995). The constitutive form of heat shock protein 70 (*hsc*70) is also well documented. For example, field transplant experiments on *Goniopora djiboutiensis* (Vaughan, 1907) demonstrated elevated levels of *hsc* and/or *hsp*70 following increased thermal shock (Sharp and others 1997); but many studies identifying a 70 kDa stress protein have failed to differentiate between constitutive and inducible forms (Robbart and others 2004; Robbart 2002; Thompson and others 2001). Differentiation between these two forms is of interest, as the increased synthesis of the constitutive *hsc*70 may act to stabilize proteins prior to the induction of an inducible isoform such as *hsp*70 during the initial stages of a stress event (Robbart and others 2004; Thompson and Scordilis 2001). Elevated levels of *hsc*70 were detected in mice 15 min after exercise stress, suggesting this so-called constitutive protein was acting as a "first defender" following the induction of a stress event (Robbart and others 2004; Thompson and Scordilis 2001).

### *Water flow and the Synthesis of hsps*

This study was specifically designed to address the effects of water-flow on coral bleaching using *in situ* heated flow chambers. As introduced in Chapter 1, water flow is beneficial to corals during periods of oxygen toxicity, and can modulate photosynthetic efficiency, that is QY. Little, if anything, is known about the distribution of molecular

chaperones within coral colonies in high water-flow environments. Would a high flow environment and a coincident thermal increase result in an asymmetric expression of stress protein synthesis similar to the expression of QY (*cf.* Chapter 1), and therefore challenge the accepted paradigm of the metabolic advantages of increased water-flow to coral reefs?

During bleaching events, corals exposed to well-mixed flow regimes *e.g.*, due to local bathymetry, or reduced temperatures *e.g.*, through bathymetry or depth, may acquire an enhanced metabolic state and be better placed to regulate stress proteins synthesis in order to maintain normal cellular functions such as photosynthesis. We speculate that corals located in other areas on the same reef may experience micro-environmental conditions too intense to maintain an adequate synthesis of molecular chaperones, which requires additional metabolic energy (Feder and Hofmann 1999). Thus, flow-stress may manifest as an asymmetric pattern of stress protein synthesis across the coral colony relative to flow, and similar to that of QY (*cf.* Chapter 1).

Several stress proteins were studied. The constitutive form, *hsc*70, was investigated to identify an early/initial response, and *hsp70* to identify up-regulated/sustained expression of stress protein synthesis similar to that found in other studies (Robbart and others 2004; Thompson and Scordilis 2001; Thompson and others 2001). The potential co-expression of *hsp*90 was also studied, given its similar molecular functions (Lindquist 1988). The primary focus of this study was (i) to establish and maintain six individual coral colonies of *M. annularis* in self-contained coral microcosms

during two ten-day saturation missions, (ii) to remove a daily time-series of single coral polyps from different flow environments across the individual colonies, and (iii) following protein recovery and quantification (*cf.* Chapter 2), to resolve potential spatiotemporal effects that an enhanced water-flow may impose on the regulation of stress proteins, across the upstream and downstream sectors of the colonies.

# MATERIALS AND METHODS

## *Study Location and Underwater Laboratory*

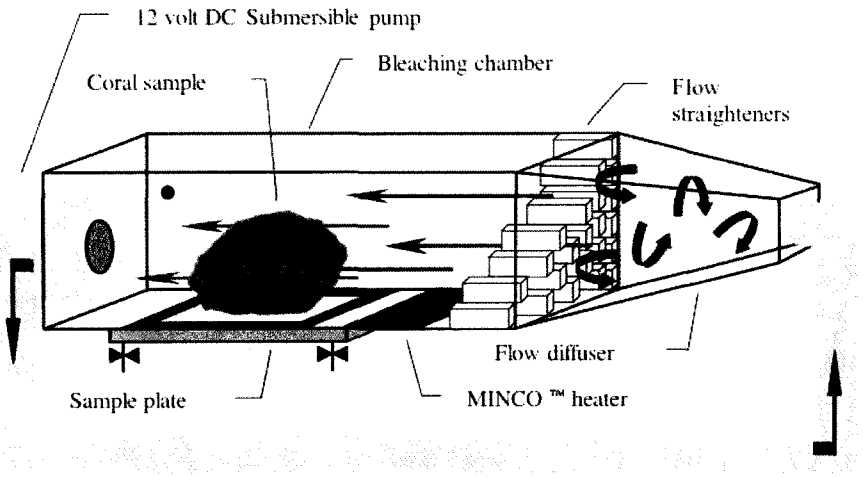
The *in situ* studies described here were conducted at the NOAA Aquarius underwater habitat located within the Florida Keys National Marine Sanctuary at 24° 57.230' N, 80° 27.223' W, during two (ten day) saturation missions in November 2002 and July 2003. On a daily basis, four saturated divers carried out several sampling methods. The nearby reef is characterized by spur and groove formations to depths of 40 m and is the focus of a wide range of research.

## *Coral Bleaching Flow Chambers*

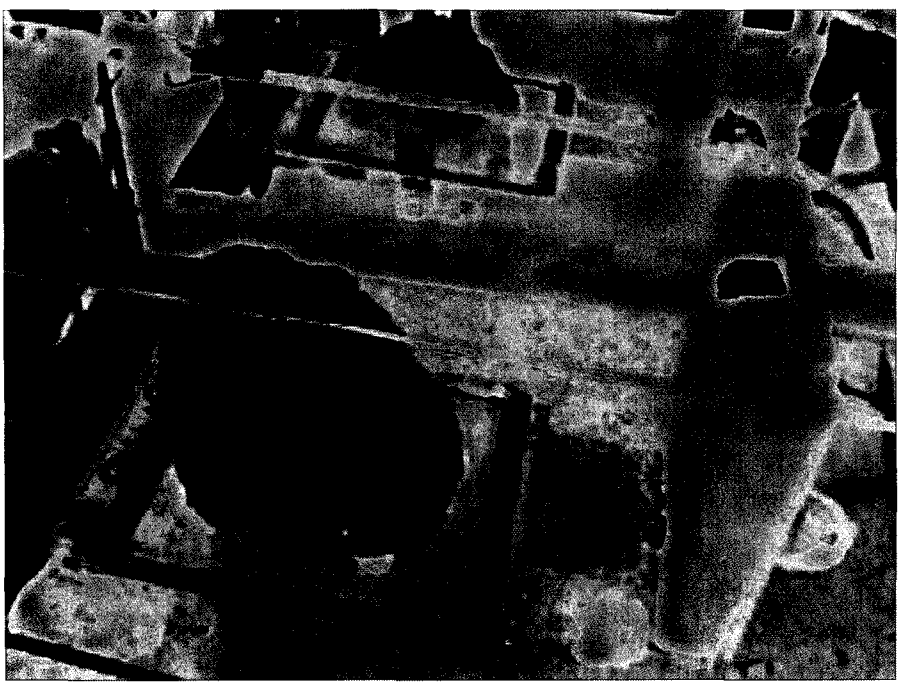
Six (7.1 L) uni-directional re-circulating coral bleaching flow chambers fabricated from 6.4 mm Plexiglas were modified from the original design (Patterson and others 1991) (*cf.* Figure 2 A.). Water flow enters the main body of the chamber via an upstream diffusing cone at speeds of *ca.* 8 - 10 cm $s^{-1}$ (Patterson and others 1991). The angle of the cone was chosen to minimize flow separation. The flow passes through a bank of rigid plastic flow straightners (i.d., 5 mm; length, 80 mm) and passes over the coral sample which is held in position on a removable Plexiglas plate with an inert sealing compound (Mortite) (Patterson and others 1991) (*cf.* Figure 2 B.).

**Figure 2 A.** Coral bleaching flow chamber design. **B.** Screenshot of two chambers with samples *in situ*. Minco Thermofoils heaters with embedded RTD sensor were located upstream of all six coral samples and in conjunction with heating from turbulence decay, provided a controlled temperature increase (1.5 - 2.0° C) above the ambient temperatures on the reef. Each heater was connected to a separate Minco PID temperature controller inside the habitat. The sample plate allowed for daily collection of QY measurements using a Diving PAM fluorometer. (*cf.* Chapter 3) and coral polyps for heat shock protein analysis.

A




B



This plate allowed for the daily removal of the coral colony reducing the stress from over handling.

Aquarius supplied electrical power to the pumps and heaters. Re-circulating flow within each chamber was provided by a 13.8 VDC, 7 Amp 1500 GPH (6300 L $hr^{-1}$) Rule bilge pumps mounted externally on the downstream side of the chambers. The water flow was returned to the upstream diffusing cone via a system of external PVC tubing (o.d. 35 mm; i.d. 28 mm). Minco Thermofoil heaters (ASI 5902 R9.50 PFB) were fixed to the floors of all six bleaching chambers upstream of the coral samples. An integrated platinum resistance temperature device (RTD) combined with the natural decay of the turbulence of the flow passing through the chamber plumbing, maintained the water temperature within each chamber at *ca.* 1.5 – 2 °C above ambient. The water temperature of each chamber was monitored and controlled from inside Aquarius, via a Minco (CT16A2020) PID temperature controller and associated Crydom 20 Amp (AC1009) solid-state relay.

Accuracy of the chamber temperature controllers was confirmed daily with a hand-held alcohol thermometer (resolution of 0.1° C), through several small access ports along the length of the chambers. Given the high flow output of the Rule pumps and vigorous mixing, no temperature gradient was detected along the length of the chambers. During the course of the experiment, ambient temperatures fluctuated on the order of 1.5° C and these fluctuations were reflected, although delayed, in the record of chamber temperatures. During the 2002 field season, chamber heaters partially failed midway through the mission, and thus the heating effect was diminished by *ca.* 1° C for some

chambers during the last three days. All chambers, however, consistently showed readings *ca.* 1.0 - 1.5° C above ambient during the entire 2002 deployment, in part due to turbulence decay. While saturated, aquanauts have few restrictions on their dive-times and were able to monitor, repair, sample, photograph and maintain any chamber and coral sample independently of the other experimental set-ups.

***Coral samples***

The subject species was the hermatypic scleractinian coral *M. annularis*, the predominant reef-building species throughout the Caribbean and the Florida Keys. Prior to the 2002 fieldwork, collection permits were obtained from the Florida Keys National Marine Sanctuary to harvest 12 (*ca.* 150 mm x 150 mm x 150 mm) mounding and flat plate morphologies of the hard coral *M. annularis*, within 1.6 km of Aquarius. A minimum cut-off depth of *ca.* 18 m ensured the collection of the correct morphotype of interest within the *Montastrea* species-complex *c.f.*, (Knowlton and others 1997; Knowlton and others 1992; Lopez and others 1999). Divers randomly identified potential samples using waterproof color-image guides. Before the removal of any sample, checks were made with a 120 x 120 mm test-frame to determine that the sample colony would physically fit into the chamber. The coral sample and surrounding area were then photographed and prevailing water flow recorded via compass bearings so that colonies could be oriented in the chamber with the dominant flow direction similar to that experienced in the field. Coral samples were cleaved off the reef substrate with a masonry hammer and a 130 mm blade-width steel chisel. Samples were transferred to the surface in individual covered containers to limit light exposure prior to the diver fully

surfacing. During transportation to the habitat, the water temperature was monitored and regulated by keeping samples out of the sun in an insulated cooler. The coral samples were re-positioned adjacent to Aquarius on the nearby sand plain at a depth of 22 m (which was within a few meters of the collection depths), and allowed to acclimate for three days prior to the start of the science mission. Corals were given an identification number that was retained for both years. Between and following the 2002 and 2003 saturation missions, all coral samples were tagged and reattached to the reef adjacent to Aquarius with Z-spar marine epoxy, photographed *in situ* and the location mapped. Thus the original coral samples were used for the 2003 season.

***Single Coral Polyp Sampling for Heat Shock Proteins***

12 polyp samples were initially harvested from the six coral colonies to record the baseline level of constitutive stress protein. Thereafter, once a day throughout both saturation missions, the Aquarius dive-team harvested single coral polyps (n = 2) from the *up* and *down-stream* facets of all coral colonies, using a stainless steel, polyp-spike similar to a fish-tagging tool as described and illustrated in Chapter 2. Daily sample positions for all corals were randomly selected via a grid reference for up and down-stream sectors. To excise coral polyps, a constant pressure (with rotation) was applied until the tip of the polyp spike bottomed-out on the underlying $CaCO_3$ skeleton. The spike was slowly withdrawn and the polyp captured in a pre-labeled (5 ml) syringe. If the tip bent or become dull it was discarded and replaced.

Once at the surface, samples were transferred into labeled cryogenic Eppendorfs centrifuge tubes containing a cocktail of proteases inhibitors (P8340, Sigma) to inhibit protein degradation (Cox and Lewis 2003; Griffin and Bhagooli 2004), diluted by volume to a ratio of 1:100 (inhibitor:sample). Daily polyp sampling was scheduled to coincide with a visit by a support vessel, and polyps were transferred to the surface via support divers. Samples were immediately stored in dark and on ice during transit to shore-based laboratory in Key Largo, transferred to liquid nitrogen and shipped back overnight to VIMS on dry ice. Samples were then stored at -80° C until analysis.

***Flow Visualization***

A visual record of the water flow over each coral sample, was made by filming hydrated brine shrimp cysts passing over each coral in its chamber. The cysts were illuminated with a modified (25 W) hand-held divers halogen lamp that cast a collimated beam of light (*ca.* 80 mm x 3 mm) perpendicular to the water-flow over the coral sample. The video was recorded using a Sony Hi 8 mm-analog camcorder in an underwater housing. The Hi 8 mm videotape was digitized using iMovie on an Apple G4 using a Sony analog Hi 8 mm control deck. For each of the sectors (n = 2) (upstream, downstream) and at three heights (0.3, 0.6, 1.0 cm) above the coral polyps, individual shrimp cysts (n = 3) were selected and tracked for five frames (1/6 s), then averaged. Within all coral samples, speeds measured within the downstream sector appeared less than 7.3 cm $s^{-1}$. Due to the highly turbulent nature of the downstream sector (d), the speeds reported elsewhere in a related study (*cf.* Chapter 2) are a snapshot, and do not show an increase of speed with height over the colony. Flow speeds and turbulence

values are reported elsewhere for this apparatus (cf. Chapter 1). For this study, shear rates at the various locations were calculated and are expressed in reciprocal seconds, derived from the relationship, dU/dz, where dU is the difference in velocity between two points spaced dz apart in the vertical direction (Table 1).

**Table 1.** The shear rates on the upstream and downstream sides of corals in the bleaching flow chambers (n = 6) (2002) were measured by tracking brine shrimp cysts using frame-by-frame video analysis in a collimated beam of light over coral colonies.

| | **Coral colony identifier** | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| **Sector** | **Shear rate ($s^{-1}$)** | | | | | |
| **Upstream** | 36.0 | 45.0 | 45.0 | 30.0 | 45.0 | 45.0 |
| **Downstream** | 5.4 | 4.2 | 3.6 | 2.4 | 9.0 | 7.2 |

***Protein Recovery and Laboratory analyses***

***Stage (i) - Protein Recovery***

A maximum of 10 polyp samples per analysis session were rapidly thawed and stored on ice, and then immediately transferred to 1.5 ml Eppendorf centrifuge tubes. Two volumes (of the polyp sample) of Laemmli reducing buffer (minus glycerol and bromophenol blue) were added to each Eppendorf centrifuge tube (Laemmli 1970). Polyps were ground for 60 seconds with a Teflon Eppendorf grinder (Kontes No. 749521-1500), whilst keeping the sample on ice. Samples were then boiled for 5 minutes, and re-ground for a further 60 seconds. Polyp samples were then spun for 15 minutes at 10,000g and the supernatant removed and aliquoted.

***Stage (ii) – Protein Purification***

Trichloroacetic acid and Deoxycholate (TCA + DOC) + *Acetone* + *2-mercaptoethanol*: Four volumes of acetone containing 15% TCA + 1% DOC and 0.07% 2ME was added to the polyp homogenate, agitated and incubated for 3 hours at -20 °C. Following incubation, the homogenates were centrifuged at 15,000g for 20 minutes and the supernatant was removed from the pellet and discarded. The pellet was vortexed in 500 µl of ice-cold (-20° C) acetone containing 0.07% 2ME and centrifuged at 15,000g for 20 minutes. The supernatant was again removed and discarded and this process repeated two further times. The pellet was allowed to air dry for 10 minutes at room temperature, then re-suspended in 25 µl of 8M urea, and 75 µl of double strength Laemmli sample buffer, and if necessary agitated and re-heated. A 6 µl sample was drawn off for protein

quantification and the remaining solution was stored at -20° C until required for further analysis.

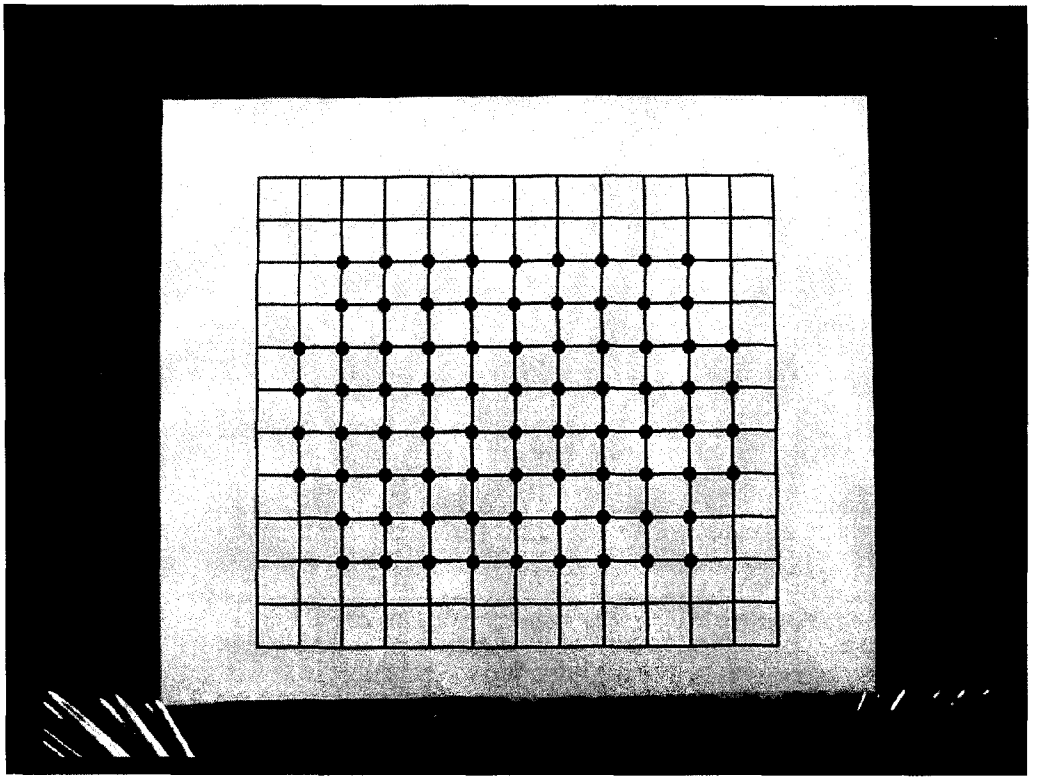
***Stage (iii) – Total Protein Estimation***

A new method for total protein estimation was developed to assess total coral protein from a small sub-sample volume (2 µl), at low concentrations (< 200 mg/ml) and in the presence of a Laemmli sample buffer (*cf.* Chapter 2). This total protein estimation method had to be developed, so a consistent amount of protein could be loaded into the SDS PAGE gels for the quantification of *hsps*. Immobilon-FL (Millipore) (PVDF), was activated in 100% methanol and subsequently placed in ultra-pure water for five minutes. The activated membrane was briefly dried on filter paper (Whatman) to remove surface water and then transferred directly onto dry, sterile Parafilm that was stretched and secured over a 1 $cm^2$ grid. Before the membrane dried, 2 µl aliquots of each protein sample were added in duplicate to the membrane using the underlying grid as a guide. For protein estimation, a logarithmic dilution series of (3.12, 6.25, 12.5, 25, 50, 100, 200, 400, 800, and 1600 $\mu$g/ml), of acetone precipitated bovine serum albumin (BSA) in double strength reducing sample buffer (2 µl) was also loaded onto the membrane. Following the application of samples and dilution series, the PVDF was air dried to allow adhesion of the proteins, then reactivated in methanol and washed 3 times (5 minutes each) in ultra-pure water. The blot was stained with freshly made Coomassie blue (G250, Sigma) for one minute, and then de-stained in the dark with multiple changes of de-stainer (10% acetic acid, 50% methanol, 40% water) until the background was white. The de-stained PVDF was allowed to dry, placed face down on the surface of the

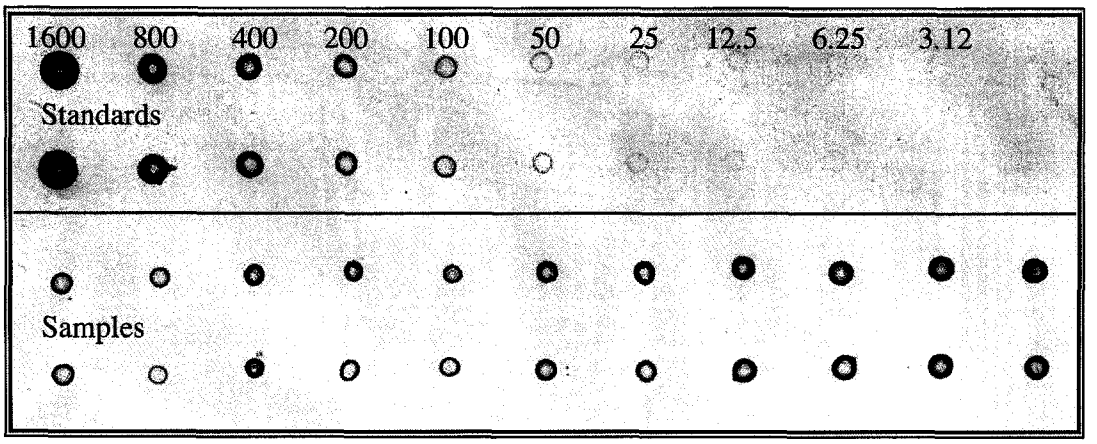
Odyssey Infrared Imaging System (LI-COR) and then scanned in the 700 nm channel with a 0.00 mm focus offset at 84 μm/pixel resolution (*cf.* Figure 3 A & B).

**Figure 3 A.** The activated membrane was briefly dried on filter paper (Whatman) to remove surface water and placed directly onto a piece of dry and sterile Parafilm, stretched over a 1 $cm^2$ grid to guide placement of 2 µl aliquots. **B.** Prior to the membrane drying out, 2 µl aliquots of each protein sample were added in duplicate to the membrane (bottom two rows) using the underlying grid as a guide. For protein estimation a dilution series of acetone precipitated bovine serum albumin (BSA) in double strength reducing sample buffer was added in duplicate to the membrane (top two rows) (Laemmli 1970). The de-stained PVDF was allowed to dry, placed face down on the surface of the Odyssey Infrared Imaging System (LI-COR) and scanned in the 700 nm channel with a 0.00 mm focus offset at 84 µm/pixel resolution.

A



B



***Stage (iv) - SDS Polyacyrlamide Gel Electrophoresis, Western blotting and Heat Shock Protein quantification***

A number of 10 % SDS-PAGE gels (Harlow and Lane 1988), were loaded with a broad ranged pre-stained molecular weight marker (BioRad), and 10 ng of *hsp*70, *hsp*90, and *hsc*70 (NSP-555, SPA-770 and SPA-751, Stressgen Bioreagents) to serve as a positive control and a calibrant for gel quantification. The remaining wells were loaded with the equivalent of 5 μg of each coral polyp homogenate, computed from the quantification of total protein. The proteins were electrophoresed at 125 VDC until the dye front reached the bottom of the gel. The contents of the gel were transferred to PVDF as per the manufacturer's instructions. The membranes were blocked with 3% casein for one hour with shaking, after which the membranes were then incubated overnight at 4° C with 20 ml of a 0.5 μg/ml solution of primary antibody, washed three times with phosphate buffered saline (PBS), then incubated for one hour with the appropriate secondary antibody (*cf.* Table 2) and washed again. The wet membranes were scanned on the Odyssey Infrared Imaging System (LI-COR) in both the 700 and 800 nm channel with a 0.00 mm focus offset at 84 μm/pixel resolution. For details of validation methods performed on all methods (*stages i – v*)
*cf.* Chapter 2.

**Table 2.** Details of the three positive control/calibrants for *hsp*, primary antibodies and fluorescent secondary antibodies, used in concert with the Odyssey Infrared Imaging System (LI-COR).

| Positive control/calibrants | Primary Antibody | Secondary Antibody |
|---|---|---|
| **Human anti-*hsp*70** (NSP-555 Stressgen) | **Rabbit *hsp*70 Protein** (SPA-812 Stressgen) | **Anti-Rabbit IR 680** (Molecular Probes) |
| **Bovine *hsc*70 Protein** (SPA-751 Stressgen) | **Rat anti-*hsc*70** (SPA-815 Stressgen) | **Anti-Rat IR800** (Jackson ImmunoResearch) |
| **Human *hsp*90 Protein** (SPA-770 Stressgen) | **Rat anti-*hsp*90** (SPA-840 Stressgen) | **Anti-Rat IR800** (Jackson ImmunoResearch) |

# RESULTS

## *Statistical analysis*

The 2002 and 2003 data sets for heat shock protein 70 (*hsp*70), the constitutive form of heat shock protein 70 (*hsc*70), and heat shock protein 90 (*hsp*90), were analyzed through a repeated measure analysis of covariance (ANCOVA) (Kutner and others 2005), to determine if significant differences existed between areas of high and low water-flow during the two (8 day - 2002; 9 day - 2003) treatments. These analyses were performed using μg/μl of the protein of interest (calculated from the protein quantification analysis), as the response variable, 'year' (n = 2), 'colonies' (n = 5) and 'sectors' (upstream, downstream) as factors, and 'days' as the covariate. All data were first $\log_{10}$ transformed to generate a normal distribution for statistical analysis and then back-transformed for graphing (Kutner and others 2005). Statistical analyses were performed using MINITAB (version 14). For a summary of ANCOVA results see Table 3, and for an example of a typical ANCOVA output (2003) see Table 4.

**Table 3.** Summary of ANCOVA results for the expression of heat shock protein 70 (*hsp*70), the constitutive form *hsc*70 and *hsp*90 over both years. Note the effect of coral 'colony' was not significant, but the effect of 'sector' (upstream vs. downstream) and 'day' were significant in both years. Chamber temperature had a significant effect in 2002, not 2003, when better temperature regulation was achieved. All statistical analyses were performed using MINITAB (version 14).

| | **Heat shock Protein *P* values** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Parameter** | | ***Hsp*70** | | | ***Hsc*70** | | | ***Hsp*90** | |
| | **2002** | **2003** | **2002/3** | **2002** | **2003** | **2002/3** | **2002** | **2003** | **2002/3** |
| **Year** | n/s | n/s | n/s | n/s | n/s | n/a | n/s | n/s | n/a |
| **Coral-colony** | n/s | n/s | n/s | n/s | n/s | n/a | n/s | n/s | n/a |
| **Sector** | *0.021* | *< 0.0005* | *0.003* | n/s | n/s | n/a | n/s | n/s | n/a |
| **Day** | *0.009* | *0.011* | *< 0.0005* | n/s | n/s | n/a | n/s | n/s | n/a |
| | **Temperature *P* values** | | | | | | | | |
| **Chamber** | *0.013* | n.s. | n.a. | *0.013* | n.s. | n.a. | n.a. | n.a. | n.a. |

n/s = not significant, n/a = not applicable

**Table 4.** General linear model; $\log_{10}$ expression of heat shock protein 70 (*hsp*70) versus 'year', 'colony' and 'sector' (upstream, downstream). Summary (example) of ANCOVA results for the pooled data of *hsp*70 quantification (2002 and 2003), including the identification of key statistical elements. All statistical analyses were performed using MINITAB (version 14).

| Factor | Type | Levels | Values |
|---|---|---|---|
| Year | Fixed | 2 | 2002, 2003 |
| Colony | Fixed | 5 | 1, 3, 4, 5, 6 |
| Sectors | Fixed | 2 | Up & Downstream |

**Analysis of Variance for $\log_{10}$ *hsp*70 quantification using Adjusted SS for Tests**

| Source | DF | F | P value |
|---|---|---|---|
| Day | 1 | 36.87 | *< 0.0005* |
| Year | 1 | 0.26 | *0.612* |
| Colony | 5 | 0.86 | *0.510* |
| Sectors | 1 | 9.00 | *0.003* |

| Term | Coef | SE Coef | T | P value |
|---|---|---|---|---|
| Constant | 0.87674 | 0.09858 | 8.89 | *< 0.0005* |
| Day | -0.09182 | 0.01512 | -6.07 | *< 0.0005* |

***Heat Shock Protein 70, 90and hsc70 expression* (2002 & 2003)**

The data sets for the constitutive form of heat shock protein 70, *hsp*70 and *hsp*90 (2002 and 2003), were initially analyzed separately. The effect of the covariate 'day' was statistically significant for *hsp*70 (2002 and 2003). 'Day' was not significant for *hsc*70 and *hsp*90 (2002 and 2003). The factors 'year', 'colony' and 'sectors' were included within all stress protein analyses, with only 'sectors' being statistically significant for *hsp*70 (2002 and 2003), but not for *hsc*70 and *hsp*90. We shall from here on only report on *hsp*70. The expression of *hsp*70 was collectively re-analyzed within the three factors: 'year' (n = 2), 'sectors' (n = 2) and 'colonies' (n = 5), during both 8 and 9-day treatment periods (2002 and 2003, respectively). A repeated-measures ANCOVA (Kutner and others 2005) resolved significant differences within the expression of *hsp*70, as an asymmetric pattern across the coral's surface in addition to the response occurring at different times of the year (November 2002 and July 2003).

***Analysis of Heat Shock Protein* 70 *Expression* (2002 & 2003)**

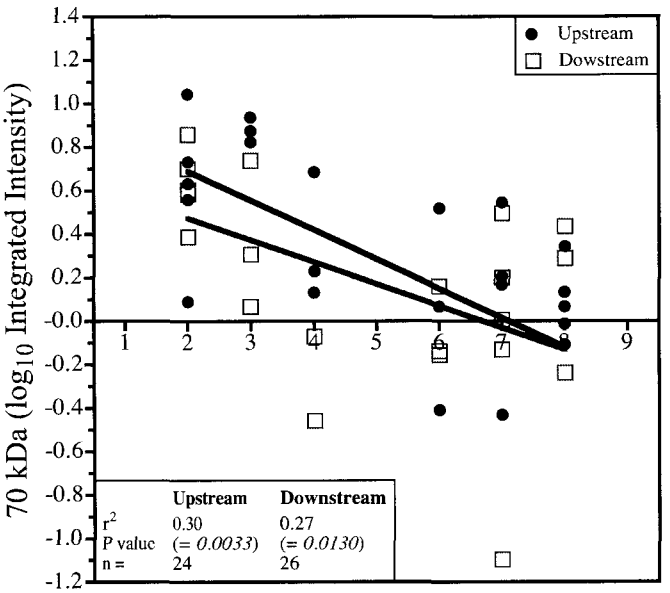
The covariate 'day' was significant in both the individual year analysis ($p = 0.009$) for 2002 and ($p = 0.011$) for 2003), as well as in the pooled data ($p < 0.0005$). The factor 'sector' was significant in both the individual year analysis ($p = 0.021$) for 2002 and ($< 0.0005$) for 2003, as well as the pooled data ($p = 0.003$), with upstream sectors having higher hsp70 expression. The factors 'year' and 'colony' were not significant in either the individual years, or the pooled data (2002 and 2003).

***Heat Shock Protein Expression and Day (2002 & 2003)***

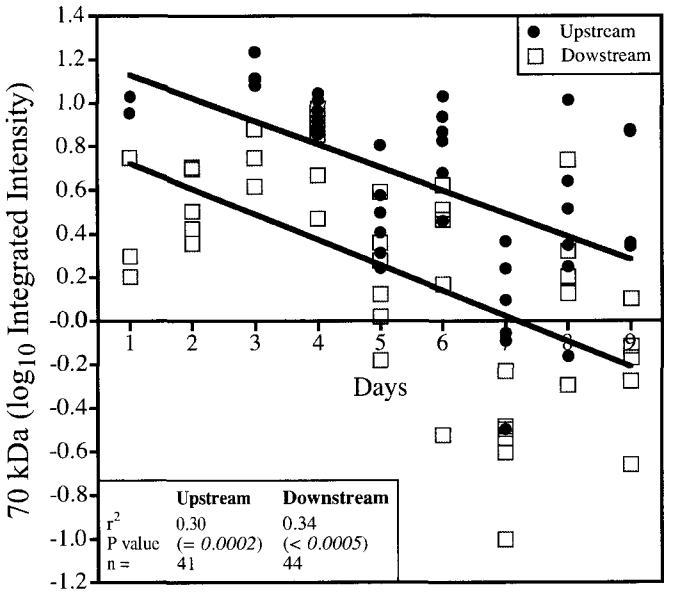
A regression analysis resolved *hsp*70 expression to be dependent on day (*cf.* Figure 4 A & B).

**Figure 4.** The relationship between day, upstream and downstream sectors, and heat shock protein expression, (*hsp*70) in colonies of *M. annularis* in flow chambers during 2002 (**A**) and 2003 (**B**). Note the statistically significant inverse relationship in both years. Two *in situ* field experiments using coral bleaching flow chambers with increased flows (*ca.* 40 cm $s^{-1}$) and elevated temperatures (*ca.* 2° C above ambient) within coral colonies (n = 5) of *M. annularis*. The synthesis of *hsp*70 was up-regulated within increased velocity gradients on the upstream sectors of the coral. Data was $\log_{10}$ transformed prior to computations and graphical portrayal. All statistical analyses were performed using MINITAB (version 14).

A




B



## DISCUSSION

This study is the first to investigate flow-modulated asymmetric patterns of stress protein regulation within mounding and flat plate morphologies of *M. annularis* following periods of thermal stress and elevated water-flow. Using *in situ* unidirectional flow chambers, elevated temperature and daily polyp removal, the same coral colonies (n = 5), were treated over two field seasons (July 2002 and November 2003). Significant elevated levels of *hsp*70 expression were resolved over upstream sectors (faster water flow) of all manipulated coral colonies during both field seasons, compared to downstream sectors (slower water-flow) (*cf.* Figure 5). There was no statistically significant up/down regulation within the coral colonies during both field seasons, in either constitutive heat shock protein (*hsc*70) or heat shock protein 90 (*hsp*90) (*cf.* Figure 6). These results suggest the physical location of both elevated and reduced stress protein regulation within individual treatments, appears modulated by water-flow with the highest protein expression located across the upstream positions. Given the different morphotypes and overall dimensions of the individual coral colonies, we would anticipate the water-flow dynamics within each chamber to be unique to that particular coral/chamber combination. It is therefore of interest that all the corals (at least at the cellular level) appear to be responding in a similar fashion.

**Figure 5.** The daily up and downstream expression of heat shock protein 70 (*hsp70*), during two *in situ* field experiments using coral bleaching flow chambers with increased flows (*ca.* 40 cm $s^{-1}$) and elevated temperatures (*ca.* 1.5 - 2° C) above ambient, within coral colonies (n = 5) of *M. annularis*, during 2002 (**A**) and 2003 (**B**). Data were pooled for up and downstream sectors (n = 2) per day in each year (n = 8 & n = 9). *Error bars* are standard errors of the mean as reported in the analysis of covariance. Means represent the average of each sector per day in all treated corals. Data were $\log_{10}$ transformed prior to statistical computations and back-transformed for graphical portrayal. All upstream samples material for day 2 in 2003 was lost whilst in storage. All statistical analysis was performed using MINITAB (version 14).

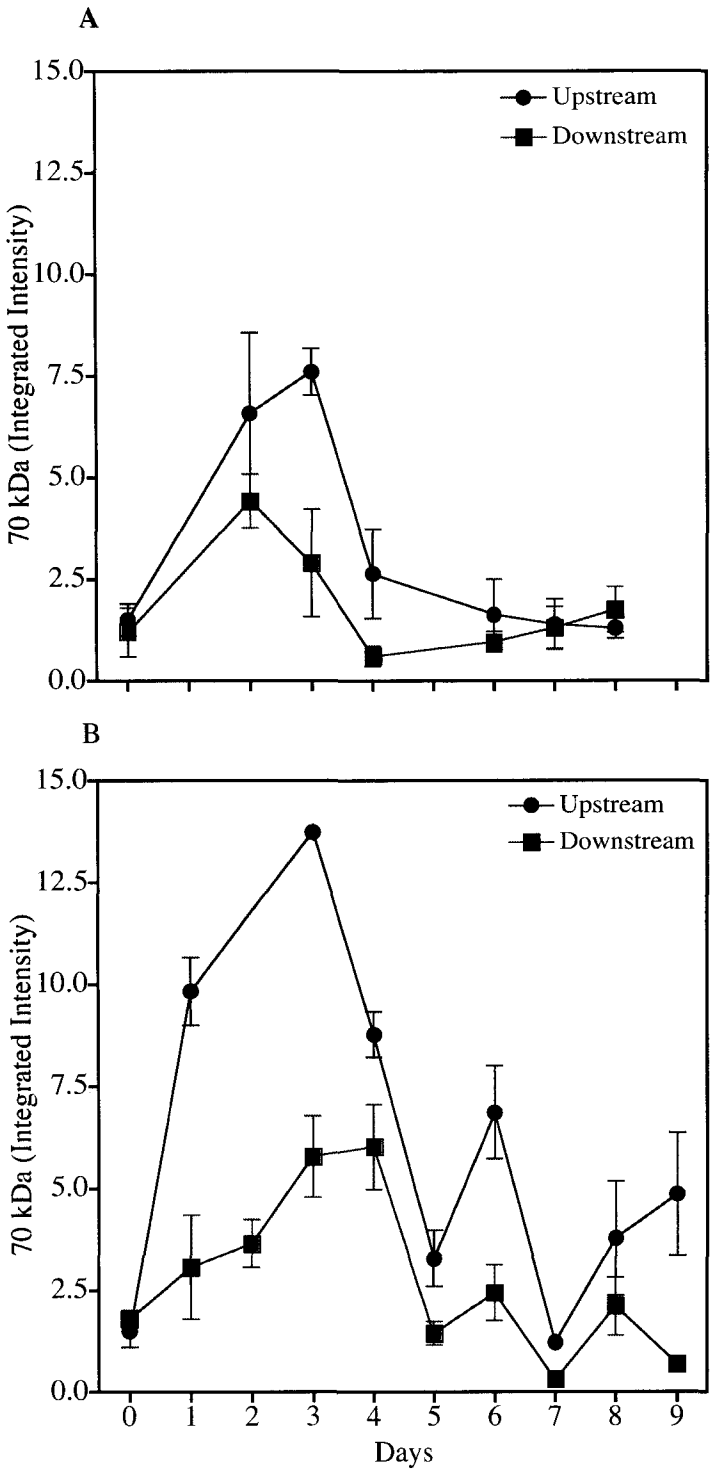
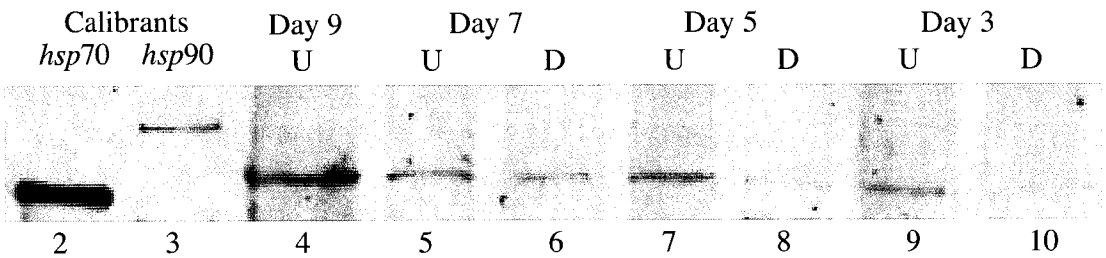


**Figure 6.**

The simultaneous detection of heat shock protein 70 (*hsp*70) and heat shock protein 90 (*hsp*90) was resolved via western blotting, using the Odyssey Infrared Imaging System (LI-COR). Sample shown is representative. The equivalent of 5 μg of total protein from up and downstream sectors of coral # 3, days 3, 5, 7 and 9 of 2003, were added to lane numbers 4 through 10 and assessed by western blot, and the 700 nm (red - *hsp*70) and 800 nm (green - *hsp*90) channels. Significant elevated levels of *hsp*70 expression were resolved over upstream sectors (faster water flow) of all manipulated coral colonies (Day 9, downstream (D) not shown) during both field seasons, compared to downstream sectors (slower water-flow). There was no statistically significant up/downstream regulation within any coral colonies during both field seasons, in either *hsp*90 or the constitutive heat shock protein 70 (*hsc*70) (not shown).



Throughout the course of the experiment, daily digital images recorded a flow-modulated bleaching-effect across the upstream sectors of all coral colonies in both years. After eight days (2002), sections of the upstream 'raised leading-edges' had visible pigmentation loss, a symptom of coral bleaching on areas of increased acceleration due to colony profile and accelerated chamber flow. The water-flow régimes within the reef-slopes of the Florida Keys include high frequency inputs from internal waves that contain plankton and dissolved nutrients, increased dissolved oxygen and enhanced flow rates (Leichter and Miller 1999; Leichter and others 2003). A growing body of evidence supports the hypothesis that enhanced water-flow prevents the accumulation of damaging oxygen byproducts in the soft tissue of coral, this oxygen being a byproduct of photosynthesis (Finelli and others 2006; Lesser 1996; Lesser and others 1994; Nakamura and van Woesik 2001; Nakamura and others 2003; Patterson 1992). Several studies have now focused on water-flow, coral reef bleaching and enhanced rates of resistance and recovery to bleaching events (Done and others 2003; Finelli and others 2006; Nakamura and van Woesik 2001; Nakamura and others 2003; West and Salm 2003). Nakamura and van Woesik (2001) have suggested that moderate to high water-flow rates may facilitate partial recovery from a bleaching event, and also that the mechanisms which lead to the dysfunction of the algal-coral symbiosis in the first instance, are driven by the limiting processes of mass transfer. Whether this observed flow-effect occurs indirectly through temperature anomalies or directly through the modulation of gas exchange still needs to be addressed (Finelli and others 2006; Nakamura and van Woesik 2001).

Acclimation or a "form of experience" has been studied in the variation of a bleaching-response within the massive coral species, *Goniastrea aspera* (Verrill 1905) (Brown and others 2002a). Studies have now identified that corals (including their symbionts) may be capable of selective adaptation and acclimatization to elevated temperatures and have already produced bleaching resistant coral populations. There are potential mechanisms that may provide resistance and protection to elevated temperature and light. These include inducible *hsps* that act in refolding denatured structural and cellular proteins and the production of oxidative enzymes that disable harmful oxygen radicals (Brown and others 2002b; Coles and Brown 2003).

The advantages of the heat-shock response suggest that natural selection would maximize the expression of *hsps*. But instead, the genes encoding *hsps* have as yet to undergo unlimited amplification in the genome, and the *hsps* themselves are subjected to auto regulation by multiple molecular mechanisms (Feder and Hofmann 1999; Linquist 1993). These finding suggest, however, that *hsps* can have both positive and deleterious impacts on fitness, and that natural selection may have balanced these impacts in pre-setting the level of *hsp* expression. These negative effects may have at least two non-exclusive explanations (Feder and Hofmann 1999; Hofmann 1995a; Hofmann 1995b; Krebs and Feder 1997): Firstly, *hsps* at high concentrations may be toxic and could directly interfere with on going cellular processes (Feder and Hofmann 1999; Linquist 1993). Secondly, the synthesis and degradation of *hsps* may consume an unbearably large fraction of a cell's or organism's energy and nutrient stores, and/or occupy a sizable fraction of the synthetic/catabolic apparatus, much to the detriment of processing other

essential bio-molecules (Calow 1991; Feder and Hofmann 1999; Hofmann 1995a). The response of corals to environmental pressures, as with most organisms, reflects the frequency, severity and duration of the disturbance. Therefore, the influence of environmental changes on physiological processes will range from slight, in response to frequent and/or minor changes, to severe, in response to more extreme changes (Gates and Edmunds 1999). These environmental fluxes, provide general examples of *stress responses* where *stress* represents a reduction in fitness as a direct result of the impairment of structure and function by exogenous factors (Calow 1989; Gates and Edmunds 1999). This situation involves a net reduction in surplus energy that could be allocated to reproduction. Changes in protein metabolism required to repair and/or stabilize affected physiological processes determine some of these energetic costs, and allow the organism to acclimatize to the environmental fluxes (Hawkins 1991). Frequent and/or minor environmental change may only stimulate minor increases in energy cost, allowing biochemical repair systems to keep pace with accruing damage. More extreme conditions, such as those demonstrated by this study, may result in increased damage and/or death of the organism, due to energy constraints related to the inability to repair damage at the same rate it occurs (Gates and Edmunds 1999; Kirkwood 1981). "Protein synthesis is one of the most ATP-costly processes occurring in cells, and the synthesis and use molecular chaperones may represent a substantial – albeit as yet unquantified fraction of the cost" (Hochachka and Somero 2002)

Previous studies have shown the expression of some *hsps* can display plasticity, and that the thermal history of the individual can change the temperature threshold at

which *hsps* are induced (Buckley and Hofmann 2002; Hofmann 2005). In the present study, statistical treatments resolved a dependence on the covariate day with a negative coefficient (- 0.096), equivalent to a ~ 20% reduction (per day) in the synthesis of stress protein throughout the experiment in both upstream and downstream positions. Whether this daily trend was an expression of a flow-acclimation process and/or a thermal seasoning from prior thermal exposure, or the thermal exposure during the length of the experiment, was beyond the scope of this study. But it is interesting to consider whether a protracted treatment would have revealed a shift in the reduction of the daily protein expression, and/or an equilibration of the protein expression (between upstream and downstream positions), or ultimately a terminal bleaching effect.

The spatially asymmetric expression of stress protein raises at least two additional points of interest: the increase of protein synthesis in areas of faster water flow when compared to downstream positions, and the concept of a $FT_{max}$, (*cf.* Figure 7) above which metabolic processes may be affected. As reported in Chapter 1, photosynthesis, and potentially other metabolic diffusion processes were taking place at higher rates through thicker boundary layers at shallower velocity gradients on the downstream sectors. Within the same time period but in opposite sectors on the coral, *hsp*70 regulation was elevated and depressed respectively (*cf.* Figure 8). The concept of a $FT_{max}$, may equally apply to the modulation of *hsp* synthesis, as this study may have only observed the result of an initial response to the increased flow in the medium time-scale (days), but not to extended exposures to flow.

**Figure 7.** The proposed conceptual model of the affects of a flow threshold ($FT_{max}$) on temperature-stressed corals, contrasting QY expression (this study) and Finelli and others (2006) (*cf.* Chapter 1). A $FT_{max}$ may also apply to the modulation of *hsp* synthesis during medium exposures (days) to increased flow. Further studies using a range of slower flow speeds for longer periods would address the question of *hsp* positioning on the coral relative to a range of flow conditions and temperatures.

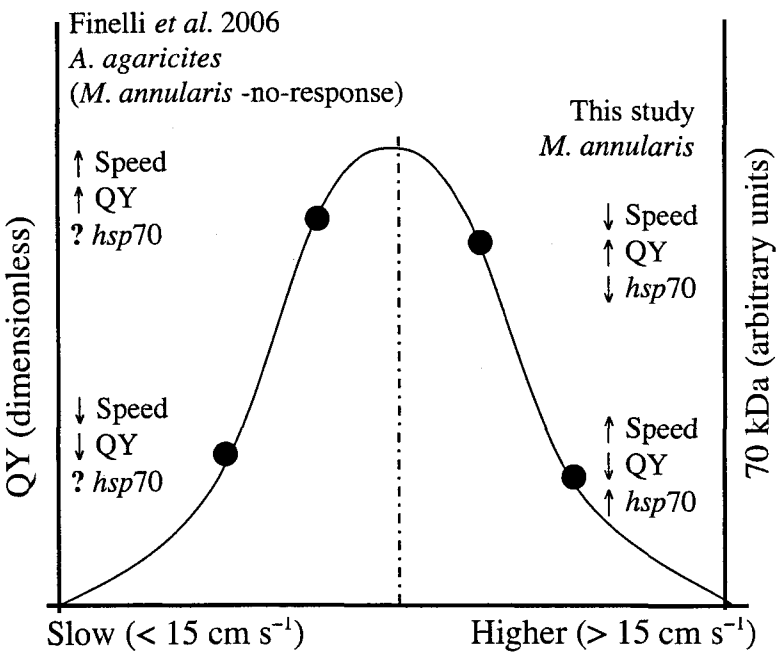
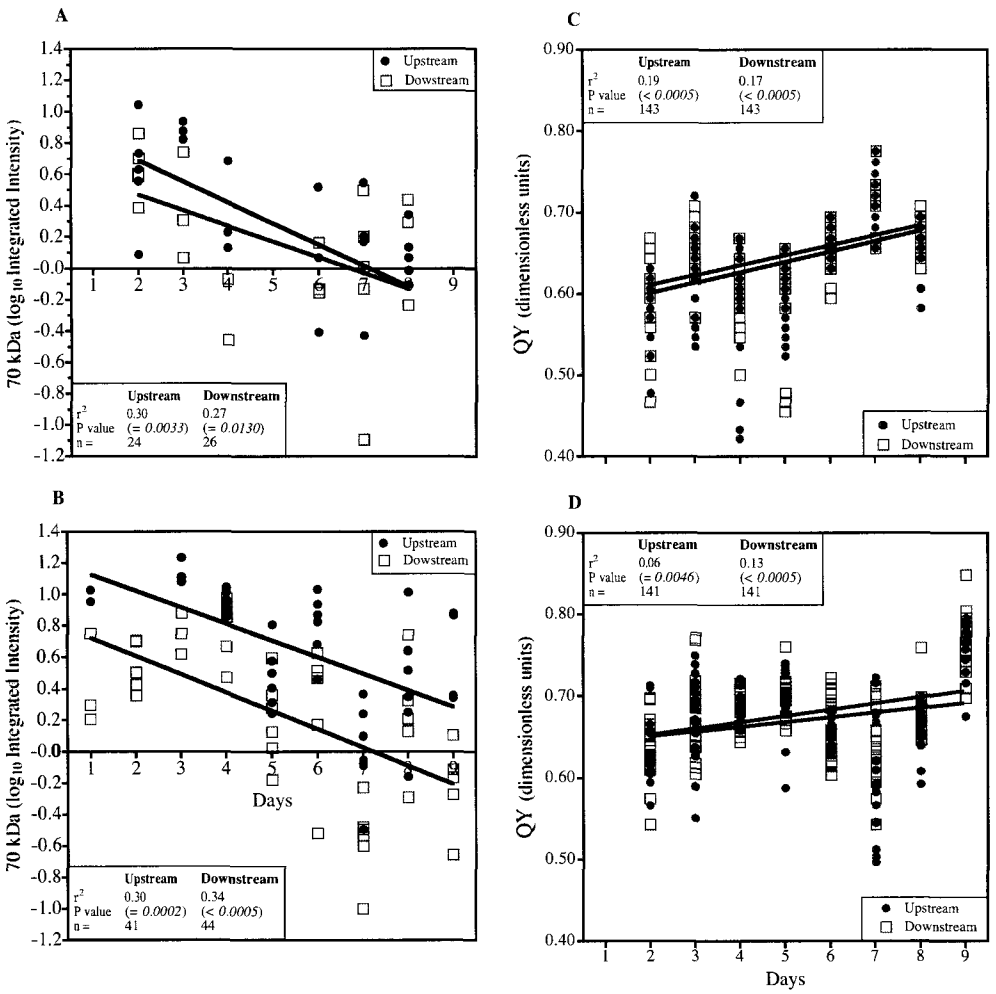


**Figure 8.** The daily up and downstream expressions of heat shock protein 70 (*hsp*70) and Quantum Yield (QY) (*cf.* Chapter 1) are compared. The relationship between day and QY, and day and *hsp*70 expression in light-adapted colonies in flow chambers during 2002 (**A & C**) and 2003 (**B & D**). Note the statistically significant inverse relationship within each example. Two *in situ* field experiments using coral bleaching flow chambers with increased flows (*ca.* 40 cm $s^{-1}$) and elevated temperatures (*ca.* 1.5 - 2° C above ambient within coral colonies (n = 5) of *M. annularis*. Photosynthetic efficiency data were arcsine transformed and *hsp*70 quantification data were $\log_{10}$ transformed, prior to statistical computations and graphical portrayal. As reported in Chapter 1, higher rates of photosynthesis were taking place at through thicker boundary layers and at depressed velocity gradients on the downstream sectors, while within the same chamber conditions, the synthesis of *hsp*70 was up-regulated within increased velocity gradients on the *upstream sectors* of the coral.



It could also be argued that *hsp*70 synthesis was depressed (in the downstream positions) due to the high rates of photosynthesis and/or, that elevated protein expression in upstream positions suppressed QY, due to energy constraints. To further elucidate these potential negative feedback processes, will require additional manipulations and time-series based sampling. Nonetheless, the concurrence of these modulated metabolic processes in opposite sectors of the coral, in concert with the novel sampling procedures introduced in Chapter 2, can now be employed in future studies.

The arrival of internal bores on the reef slope, are associated with semidiurnal internal tides, sharp temperature drops (> 5.4° C), and increased salinities (> 0.6 ‰) over short time periods of 1-20 minutes. The effects of these internal waves within the Florida reef system have been well documented (Leichter and Miller 1999; Leichter and others 1998; Leichter and others 1996; Smith 1983). These dynamic events appear regularly at Conch Reef from May through November, with the highest activity measured in July through September (Leichter and others 2003; Leichter and others 1996). In both field seasons, a number of transient thermal anomalies were observed in the daily temperature output of the bleaching chambers, in addition to divers experiencing the rapid onset of increased water flow. The effect of these random temperature anomalies may appear reflected in the pattern of *hsp*70 expression. Day two (downstream) and day three (upstream) of 2002 marks a decline in *hsp* expression and then again on day three (upstream), day four (downstream) and day six (up and downstream) of 2003 (*cf.* Figure 5). These negative trends of *hsp* expression could be interpreted a metabolic acclimation to the stress of the treatments.

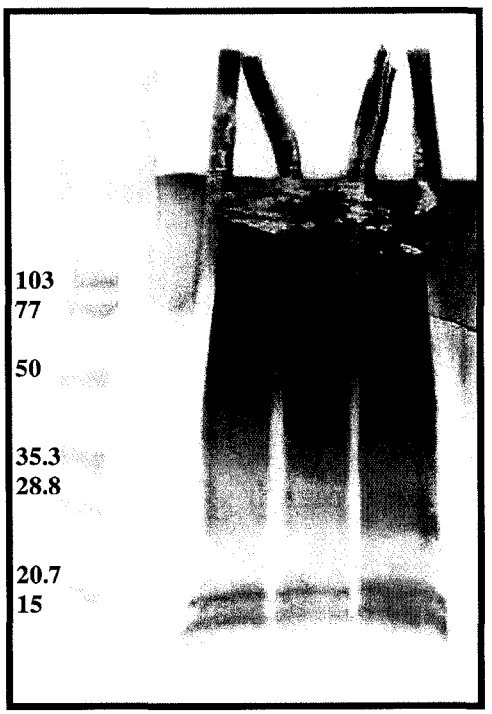
Flow regimes on coral reefs are diverse and maintained by a range of processes. Corals and other sessile reef organisms live within boundary layers that are more often than not turbulent than laminar, and the velocity profiles across these layers, as just described, rely on surface roughness (the height and shape of roughness elements), and mainstream flow above these surfaces (Sebens and others 2003; Shashar and others 1996). Both currents and waves generate substantial flow on reefs. Oscillatory flow generated by waves, that is collapsed wave orbitals, prevent the build up of a steady-state momentum boundary layer similar to those found in unidirectional flow *e.g.*, currents. Furthermore, corals on exposed (*e.g.*, convex) surfaces may experience higher flows compared to the severely reduced flows in concavities and within aggregations (Chamberlain and Graus 1975; Helmuth and others 1979; Sebens and others 2003). Within this study, the chamber-generated velocity gradients, although unidirectional, were comparable to the previously reported ambient flow speeds for the same reef system (Finelli and others 2006). It could be argued that observed differences in stress protein expression across the coral surface may instead be attributable to the unidirectional nature of the chamber flow via a hydraulic effect (for example a fluid-mechanical pressure) on the soft tissue of the coral. There are a number of mechanisms that sessile organisms can influence the magnitude of these flow-induced forces, along with the distribution and degree of the flow induced mechanical stresses experienced by the organism. Furthermore, the mechanical properties of the organism's tissues affect how much they deform and whether or not they will fail in response to flow-induced stresses (Koehl 1984). Many of these questions could be addressed with additional studies using bi and multi-directional flow-through heat/no heat chambers, fitted over *in situ* coral colonies.

***Constitutive Heat Shock Protein 70, Heat Shock Protein 90 and Cnidaria- Specific Antibodies.***

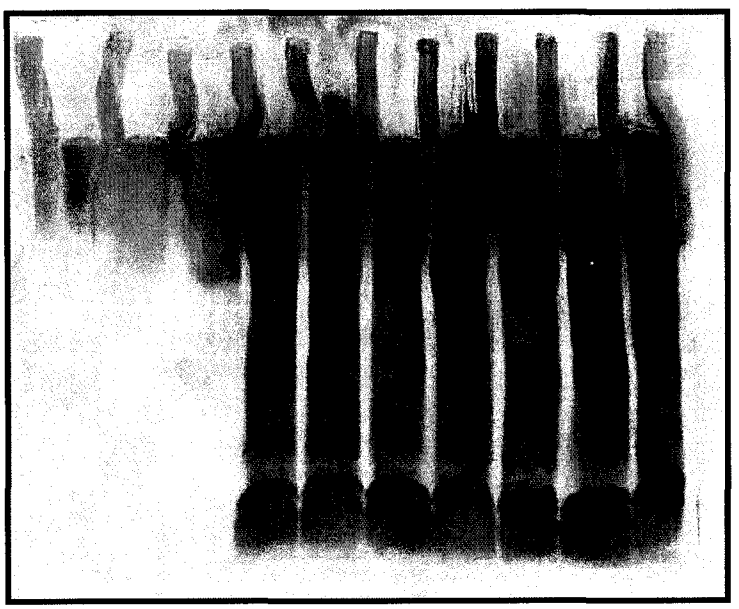
We were unable to resolve *hsp*70, following a published preparation method for coral and utilizing the only cnidarain-specific primary antibody then available (Downs and others 2000). Western blots appeared heavily contaminated with polysaccharide smearing and non-specific binding (*cf.* Figure 9 A & B) and we were frequently unable to load sufficient protein (*ca.* 40 $\mu$g) from a single coral polyp. We believe it was the low volume of one polyp that precluded us from using established and published methods. Most studies cleave large samples of coral fragments (*ca.*100 $cm^2$) from live colonies and then process the sample as a whole. We had to maintain the study-organisms for two saturations mission (10-days), as well as ensure their post fieldwork survival. Low volume sampling and therefore potentially less stress for the coral, in concert with time-series sampling appeared the only option. Therefore destructive sampling methods were not feasible. These issues early on in this study prompted the development of a single coral polyp sampling method and preparation as introduced in Chapter 2.

**Figure 9**. Two examples of initial Western blot protein profiles. These early trials were unsuccessful in resolving stress proteins in coral samples, as levels of interfering contaminants and/or non-specific binding inhibited protein resolution (dark smearing along the lanes) and protein break-down products (darker bands at bottom of the lane) (**A & B**). These results were typical after following published methods and using cnidarian-specific primary antibodies. Molecular weights of purified standard indicated in **B**.

A




B



The biotechnology industry, manufacture a range of secondary antibodies including *hsc*70, *hsp*70 and hsp90, which although (at the moment) not specific for cnidarians, have been extensively used (King and others 2002; Locke and Atance 2000; Scott and others 2003), including the field of marine biology (Chang 2005; Encomio 2004; Hamdoun and others 2003; Sorte and Hofmann 2004) and more importantly Cnidaria (Black and others 1995; Downs and others 2002; Robbart and others 2004). As *hsc*70 and *hsp*90 are extensively documented in the literature including that of *M. annularis* species complex *c.f.*, (Black and others 1995; Hayes and King 1995; Knowlton and others 1997; Knowlton and others 1992; Lopez and others 1999; Rossi and Snyder 2001), we are somewhat hesitant to speculate as to the lack of success at resolving these proteins within this study. Nonetheless, we did document a degree of overlapping detection (cross-talk) between the secondary antibodies for *hsp*70 and 90 (SPA-812 and SPA-840). Given the particular focus and procedures of the study that is mode of stress (increase flow), time frame or even the experimental approach, may not necessarily have been appropriate for the reliable expression of *hsc*70 *and hsp*90.

***Summary***

Significant elevated levels of *hsp*70 expression were resolved over all upstream sectors when compared to downstream sectors of coral colonies (n = 5), during both field seasons, with no significant findings in upstream/downstream regulation within coral colonies or field season for either *hsc*70 or *hsp*90. These results suggest the physical location of elevated and reduced stress protein synthesis, appears modulated by water flow, and that all corals treatments (at least at the cellular level), appear to have

responded in a similar fashion. These results therefore, suggest a synergistic relationship between water-flow, QY, stress protein regulation and $FT_{max}$ (*cf.* Figure 7). An alternate explanation to the observed effects may be attributable to the unidirectional nature of the chamber flow via a hydraulic effect on the soft tissue of the coral, an artifact of the experimental apparatus. Statistical treatments resolved a dependence on the covariate day with a negative coefficient (- 0.096) and implies a ~ 20% reduction (per day) of stress protein expression. With regard to the opposite positioning of increased QY compared to *hsp* expression, we suggest that *hsp*70 synthesis may have been limited in the downstream position, due to the high rates of photosynthesis, or that elevated protein expression in upstream positions suppressed QY. Either model, however, would involve complex negative feedback processes.

A reef-monitoring program consists of a selection of protocols structured around methods that together provide information for effective reef management. The actual protocols and methods that are chosen will depend upon the information needed for the specific reef, the size of the area, and the available resources of people, time, equipment and money (Hill and Wilkinson 2004). Medium sized patches (one survey-day - two divers) of separate reef sites in different parts of the reef structure (fore, back, etc.) should be considered. Special attention should be paid to reef sites that become exposed at low tide, shallow reefs and areas of increased water flow. Along with daily single coral polyp sampling of the same coral colony, managers should also consider; sea surface temperature, extent, severity and recovery of coral communities, cover, diversity, size

structure, and changes in relative abundance of growth forms as well as, identifying bleaching resilient species and sites (Hill and Wilkinson 2004).

It is now well established that increased water-flow can inhibit the build-up of oxygen and/or oxygen radicals (Downs and others 2002; Jones and others 1998; Lesser 1997; Nakamura and van Woesik 2001; Nakamura and others 2003), produce thinner boundary layers and improved mass transfer rates (Patterson 1991; Patterson and Sebens 1989). Evidence now exists that during and following sustained periods of increased flow, irradiance and thermal stress, a yet-to-be determined physiological threshold within water-flow, that is $FT_{max}$, when acting in concert with thermal stress, may disrupt metabolic processes, *e.g.*, the regulation of molecular chaperone synthesis and/or restrict photosynthetic machinery. To further elucidate these potential synergistic mechanisms would require additional *in situ* flow and temperature manipulations, time-series sampling, dose response studies and laboratory based flow-chamber studies.

## LITERATURE CITED

Banaszak AT, Trench RK. 1995. Effects of ultraviolet (UV) radiation on marine microalgal invertebrate symbioses. I. Response of the algal symbionts in culture and in hospite. Journal of Experimental Marine Biology and Ecology 194:213-232.

Ben-Haim Y, Banin E, Kushmaro A, Loya Y, Rosenberg E. 1999. Inhibition of photosynthesis and bleaching of zooxanthellae by the coral pathogen *Vibrio shiloi*. Environmental Microbiology 1:223-229.

Ben-Haim Y, Thompson FL, Thompson CC, Cnockaert MC, Hoste B, Swings J, Rosenberg E. 2003. *Vibrio coralliilyticus* sp. nov., a temperature-dependent pathogen of the coral *Pocillopora damicornis*. International Journal of Systematics Evolution and Microbiology 53:309-315.

Black NA, Voellmy R, Szmant AM. 1995. Heat shock protein induction in *Montastraea faveolata* and *Aiptasia pallida* exposed to elevated temperatures. Biological Bulletin 188:234-240.

Branton MA, MacRae T, Lipschultz F, Wells PW. 1999. Identification of small heat shock/ a-crystallin protein in the scleractinian coral *Madracis mirabills* (Duch. and Mich.). Canadian Journal of Zoology 77:675-682.

Brown BE. 1997. Coral bleaching: Causes and consequences. Coral Reefs 16(Suppl.):S129-S138.

Brown BE, Downs CA, Dunne RP, Gibb SW. 2002a. Exploring the basis of thermotolerance in the reef coral *Goniastrea aspera*. Marine Ecology Progress Series 242:119-129.

Brown BE, Dunne RP, Goodson MS, Douglas AE. 2002b. Experience shapes the susceptibility of a reef coral to bleaching. Coral Reefs 21:119-126.

Buckley BA, Hofmann GE. 2002. Thermal acclimation changes DNA-binding activity of heat shock factor 1 (HSF1) in the goby Gillichthys mirabilis: implications for plasticity in the heat-shock response in natural populations. J Exp Biol 205(20):3231-3240.

Buddemeier RW, Fautin DG. 1993. Coral bleaching as an adaptive mechanism: a testable hypothesis. BioScience 43:320-326.

Calow P. 1989. Proximate and ultimate responses to stress in biological systems. Biological Journal of the Linnaean Society 1/2:173-181.

Calow P. 1991. Physiological costs of combating chemical toxicants: ecological implications. Comparative Biochemistry and Physiology 100 C:3-6.

Chamberlain JA, Jr., Graus RR. 1975. Water flow and hydromechanical adaptations of branched reef corals. Bulletin of Marine Science 25:112-125.

Chang ES. 2005. Stressed-Out Lobsters: Crustacean Hyperglycemic Hormone and Stress Proteins Integrative and Comparative Biology:45-50.

Cole J. 2003. Dishing the dirt on coral reefs. Nature 421:705-706.

Coles SL, Brown B. 2003. Coral Bleaching – Capacity for Acclimatization and Adaptation. Advances in Marine Biology 46.

Cox EF, Lewis TL. 2003. Molecular Biology of Corals. University of Hawai'i Hawai'i Institute of Marine Biology 107 p.

Done TJ, Turak E, Wakefirld M, De'ath G, Kininmonth S, Wooldridge S, Berkelmans R, Van Oppen M, Mahoney M. 2003. Tea sting bleaching resistance hypotheses of the 2002 Great Barrier Reef bleaching event: a contribution to the Transforming Coral Reef Conservation initiative and toolkit. Australian Institute of Marine Science. Final report to The Nature Conservation.

Downs CA, Fauth JE, Halas JC, Dustan P, Bemiss J, Woodley CM. 2002. Oxidative stress and seasonal coral bleaching. Journal of Free Radical Biology and Medicine 33(4):533-543.

Downs CA, Mueller E, Phillips S, Fauth JE, Woodley CM. 2000. A molecular biomarker system for assessing the health of coral (*Montastraea faveolata*) during heat stress. Marine Biotechnology 2:533-544.

Encomio VG. 2004. A study of the eastern oyster, Crassostrea virginica: 1. Dermo tolerance, survival, growth, condition and Hsp 70 expression in different geographic stocks; heat tolerance and effects of sublethal heat shock on survival and Hsp70 expression of infected and uninfected oysters.: The College of William and Mary. . 173 p.

Fang LS, Huang SP, Lin KL. 1997. High temperature induces the synthesis of heat-shock proteins and the elevation of intracellular calcium in the coral *Acropora grandis*. Coral Reefs 16:127-131.

Feder ME, Hofmann GE. 1999. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. Annual Review of Physiology 61:243-282.

Finelli CM, Helmuth BST, Pentcheff DN, Wethey DV. 2006. Water flow influences oxygen transport and photosynthetic efficiency in corals. Coral Reefs 25:47-57.

Fitt WK, Brown BE, Warner ME, Dunne RP. 2001. Coral bleaching: Interpretation of thermal tolerance limits and thermal threeholds in tropical corals. Coral Reefs 20:51-65.

Fitt WK, Warner ME. 1995. Bleaching patterns of four species of Caribbean reef corals. Biological Bulletin 189:298-307.

Gates RD, Edmunds PJ. 1999. The Physiological Mechanisms of Acclimatization in Tropical Reef Corals. Amer. Zool. 39(1):30-43.

Gleason DF, Wellington GM. 1993. Ultraviolet radiation induces coral bleaching: evidence from field experiments. Nature 365:836-838.

Glynn PW. 1984. Widespread coral mortality and the 1982-83 El Nino warming effect. Environmental Conservation 11(2):133-146.

Glynn PW. 1988. El Nino-Southern Oscillation 1982-1983: near shore population, community, and ecosystem responses. Annual Review of Ecology and Systematics 19:309-345.

Glynn PW. 1993. Coral reef bleaching: ecological perspectives. Coral Reefs 12:1-17.

Griffin SP, Bhagooli R. 2004. Measuring antioxidant potential in corals using the FRAP assay. Journal of Experimental Marine Biology and Ecology 302:201 - 211.

Hamdoun AM, Cheney DP, Cherr GN. 2003. Phenotypic plasticity of HSP70 and HSP70 gene expression in the Pacific Oyster (*Crassostrea gigas*): Implications for thermal limits and induction of thermal tolerance. Biological Bulletin 205:160-169.

Harlow E, Lane D. 1988. Appendix 1: Electrophoresis. Antibodies a laboratory manual: Cold Springs Harbor Laboratory. p 635-59.

Hawkins AJS. 1991. Protein turnover: A functional appraisal. Functional Ecology Functional Ecology 5:222-233.

Hayes RL, King CM. 1995. Induction of 70-kD heat shock protein in scleractinian corals by elevated temperature: significance for coral bleaching. Molecular Marine Biology and Biotechnology 4(1):36-42.

Helmuth BST, Sebens KP, Daniel TL. 1979. Morphological variation in coral aggregations: branch spacing and mass flux to coral tissues. Journal of Experimental Marine Biology 209:233-259.

Hill J, Wilkinson C. 2004. Methods of Ecological Monitoring of Coral Reefs. 117 p.

Hochachka PW, Somero GN. 2002. Biochemical adaptation, mechanisms and process in physiological evolution. New York: Oxford University Press. 466 p.

Hoegh-Guldberg O. 1999. Climate change, coral bleaching and the future of the world's coral reefs. Marine and Freshwater Research 50:839-866.

Hofmann AA. 1995a. Acclimation: increasing survival at a cost. Trends in Ecology & Evolution 10:1-2.

Hofmann AA. 1995b. Species and tissue-specific synthesis patterns for heat shock proteins *hsp*70 and *hsp*90 in several marine teleost fishes. Trends in Ecology & Evolution 10:1-2.

Hofmann GE. 2005. Patterns of Hsp gene expression in ectothermic marine organisms on small to large biogeographic scales. Integrated Comparative Biology 45(2):247-255.

Hughes T. 1994. Catastrophes, phase shifts, and large-scale degradation of a Caribbean coral reef. Science 265:1547-1551.

Jokiel PL. 1977. Effects of Temperature on the Mortality and Growth of Hawaiin reef corals. Marine Biology 43:201-208.

Jokiel PL, Coles SL. 1974. Effects of heated effluent on hermatypic corals at Kahe Point, Oahu. Pacific Science 28:1-18.

Jones RJ, Hoegh-Guldberg O, Larkum AWD, Schreiber U. 1998. Temperature-induced bleaching of corals begins with impairment of the $CO_2$ fixation mechanism in zooxanthellae. Plant, Cell and Environment 21:1219-1230.

King Y-T, Lin C-S, Lin J-H, Lee W-C. 2002. Whole-body hyperthermia-induced thermotolerance is associated with the induction of Heat Shock Protein 70 in mice. J Exp Biol 205(2):273-278.

Kirkwood TBL. 1981. Repair and its evolution: Survival versus reproduction. In: Townsend CR, Calow P, editors. Physiological ecology: An evolutionary approach to resource use: Blackwell Scientific. p 165-189.

Knowlton N, Mate JL, Guzman HM, Rowan R, Jara J. 1997. Direct evidence for reproductive isolation among the three species of the *Montastraea annularis* complex in Central America (Panama and Honduras). Marine Biology 127:705-711.

Knowlton N, Weil E, Weight LA, Guzman HM. 1992. Sibling species in *Montastraea annularis*, coral bleaching, and the coral climate record. Science 255:330-333.

Koehl MAR. 1984. How Do Benthic Organisms Withstand Moving Water? Amer. Zool. 24(1):57-70.

Krebs RA, Feder ME. 1997. Deleterious Consequences of *hsp*70 over expression in *Drosophila melanogaster* larvae. Cell Stress Chaperones 2:60-71.

Kutner MH, Nachtsheim CJ, Neter J, Li W. 2005. Applied Linear Statistical Models. Gorden B, editor. New York: McGraw-Hill/Irwin. 1396 p.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Leichter JJ, Miller SL. 1999. Predicting high-frequency upwelling: Spatial and temporal patterns of temperature anomalies on a Florida coral reef. Continental Shelf Research 19:911-928.

Leichter JJ, Shellenbarger G, Genovese SJ, Wing SR. 1998. Breaking internal waves on a Florida (USA) coral reef: A plankton pump at work? Marine Ecology Progress Series 166:83-97.

Leichter JJ, Stewart HL, Miller SL. 2003. Episodic nutrient transport to Florida corals reefs. Limnology and Oceanography 48(4):1394-1407.

Leichter JJ, Wing SR, Miller SL, Denny MW. 1996. Pulsed delivery of subthermocline water to Conch Reef (Florida Keys) by internal tidal bores. Limnology and Oceanography 41(7):1490-1501.

Lesser MP. 1996. Exposure of symbiotic dinoflagellates to elevated temperatures and ultraviolet radiation causes oxidative stress and inhibits photosynthesis. Limnology and Oceanography 41:271-283.

Lesser MP. 1997. Oxidative stress causes coral bleaching during exposure to elevated temperatures. Coral Reefs 16:187-192.

Lesser MP. 2006. Oxidative Stress in Marine Environments: Biochemistry and Physiological Ecology. Annual Review of Physiology 68(1):253-278.

Lesser MP, Weis VM, Patterson MR, Jokiel PL. 1994. Effects of morphology and water motion on carbon delivery and productivity in the reef coral, *Pocillopora damicornis* (Linnaeus): Diffusion barriers, inorganic carbon limitation, and biochemical plasticity. Journal of Experimental Marine Biology and Ecology 178:153-179.

Lindquist S. 1988. The heat shock protein. Annual Review of Genetics 22:677-685.

Linquist S. 1993. Auto-regulation of the heat-shock response. Translational Regulation of Gene Expression. New York: Plenum. p 279-320.

Locke M, Atance J. 2000. The myocardial heat shock response following sodium salicylate treatment. Cell Stress and Chaperones 5(4):359-368.

Lopez JV, Kersanach R, Rehner SA, Knowlton N. 1999. Molecular determination of species boundaries in corals: Genetic analysis of the *Montastraea annularis* complex using amplified fragment length polymorphisms and a microsatellite marker. Biological Bulletin 196:80-93.

Nakamura T, van Woesik R. 2001. Water-flow rates and passive diffusion partially explain differential survival of corals during the 1998 bleaching event. Marine Ecology Progress Series 212:301-304.

Nakamura T, Yamasaki H, van Woesik R. 2003. Water flow facilitates recovery from bleaching in the coral *Stylophora pistillata*. Marine Ecology Progress Series 256:287-291.

Patterson MR. 1991. The effects of flow on polyp-level capture in an octocoral, *Alcyonium siderium*. Biological Bulletin 180:93-102.

Patterson MR. 1992. A mass transfer explanation of metabolic scaling relations in some aquatics invertebrates and algae. Science 255:1421-1423.

Patterson MR, Sebens KP, Olson RR. 1991. *In situ* measurements of flow effects on primary production and dark respiration in reef corals. Limnology and Oceanography 36(5):936-948.

Patterson MR, Sebens PK. 1989. Forced convection modulates gas exchange in cnidarians. Proceedings of the National Academy of Sciences 86:8833-8836.

Richardson LL, Goldberg WM, Kuta KG, Aronson RB, Smith GW, Ritchie KB, Halas JC, Feingold JS, Miller SL. 1998. Florida's mystery coral-killer identified. Nature 392:557-558.

Robbart M, L., Peckol P, Scordilis S, P., Curran H, A., Brown-Saracino J. 2004. Population recovery and differential heat shock protein expression for the corals

Agaricia agaricites and A. tenuifolia in Belize. Marine Ecology Progress Series 283:151-160.

Robbart ML. 2002. Population dynamics and differential heat shock protein expression in two Caribbean scleractinian corals, Agarica Agaricites and A, Tenuifolia [Masters]: Smith College. 98 p.

Rossi S, Snyder MJ. 2001. Competition for space among sessile marine invertebrates: changes in HSP70 expression in two Pacific cnidarians. Biol Bull 201(3):385-93.

Saxby T, Dennison WC, Hoegh-Guldberg O. 2003. Photosynthetic responses of the coral *Montipora digitata* to cold temperature stress. Marine Ecology Progress Series 248:85-97.

Scott MA, Locke M, Buck LT. 2003. Tissue-specific expression of inducible and constitutive Hsp70 isoforms in the western painted turtle. J Exp Biol 206(2):303-311.

Sebens KP, Helmuth BST, Carrington E, Agius B. 2003. Effects of water flow on growth and energetics of the scleractinian coral *Agaricia teniufolia* in Belize. Coral Reefs 22:35-47.

Sharp VA, Brown BE, Miller D. 1997. Heat shock protein (HSP 70) expression in the tropical reef coral *Goniopora djiboutiensis*. Journal of Thermal Biology 22(1):11-19.

Shashar N, Kinane S, Jokiel PL, Patterson M R. 1996. Hydromechanical boundary layers over a coral reef. Journal of Experimental Marine Biology and Ecology 199:17-28.

Smith NP. 1983. Temporal and Spatial Characteristics of Summer Upwelling along Florida's Atlantic Shelf. Journal of Physical Oceanography 13(9):1709-1715.

Sorte CJB, Hofmann GE. 2004. Changes in latitudes, changes in aptitudes: Nucella canaliculata(Mollusca: Gastropoda) is more stressed at its range edge. Marine Ecology Progress Series 274:263-268.

Thompson H, S., Scordilis S, P. 2001. Downhill running elicits distinct but similar protein and mRNA expression patterns of HSP 25, HSC 70 and HSP 70. Federation of American Societies for Experimental Biology 15:A417.

Thompson H, S., Scordilis S, P., P. CS. 2001. A single bout of eccentric exercise increase HSP 27 and HSC/HSP 70 in human skeletal muscle. Acta Physiologica Scandinavica 171:187-194.

Tomanek L. 2002. The heat-shock response: its variation, regulation and ecological importance in intertidal gastropods (genus *Tegula*). Integrative and Comparative Biology 42:797-807.

Tomanek L, Somero GN. 2002. Interspecific- and acclimation-induced variation in levels of heat-shock proteins 70 (hsp70) and 90 (hsp90) and heat-shock transcription factor-1 (HSF1) in congeneric marine snails (genus *Tegula*): Implications for regulation of *hsp* gene expression. The Journal of Experimental Biology 205:677-685.

West JM, Salm RV. 2003. Resistance and resilience to coral bleaching: Implications for coral reef conservation and management. Conservation Biology 17(4):956-967.

Wilkinson C. 2000. Status of coral reefs of the world. Australia: Global Coral Reef Monitoring Network.

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